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THE PRESERVATION OF YELLOW FEVER VIRUS*

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In preparation for a comparative study of the characteristics of strains of yellow fever virus from West Africa and Brazil, we found it necessary to seek a reliable method of preserving the virus over a considerable period of time. The ideal method for our purposes would be one which would permit the sending of active virus in small sealed containers on sea voyages lasting over a month, and would also allow storage in the laboratory for several months without serious loss of virulence. Such a method was found among several processes previously applied to the preservation of other viruses, and it has now been in use long enough in connection with our experiments to show that it can be depended upon.

In the first work with experimental yellow fever in monkeys, Stokes, Bauer and Hudson (1) kept the virus alive for a time by passing it directly from animal to animal. They made inoculations by taking blood from the sick monkey, adding it to a citrate solution to prevent clotting, and injecting the mixture into a healthy animal. This method would have required an excessive number of monkeys to preserve even one strain of the virus during an extended study. The interval between successive inoculations had to be short for several reasons: the citrated blood could not be stored more than a few days without danger of losing the virus, the incubation period was usually brief, and the blood for transmission was drawn as a rule on the first day of illness. The difficulty was later diminished by these investigators by transferring the infection from monkey to monkey by means of mosquitoes, and thus lengthening to several weeks the time interval between successive monkeys in a series. This method is far from ideal, however, for the mosquitoes require much care and occasionally develop a high

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mortality which might result in the loss of the yellow fever strain. The use of mosquitoes, moreover, necessitates special equipment and adds an extra hazard for the experimenters.

Sellards (2) succeeded in preserving yellow fever virus in frozen monkey liver for twelve days while transporting it from West Africa to England. Keeping tissues in a frozen state requires conditions difficult to arrange, especially on long voyages. The method, however, was one which promised to be highly useful, if tests proved it reliable for extensive periods, especially where the use of mosquitoes is impracticable.

The Yellow Fever Strains Used

For the purpose of comparing the values of different methods of preserving yellow fever virus we have brought together in tables the results of our observations with two strains which proved to be practically identical in their characteristics and very satisfactory for experimental work. Both were highly virulent and fairly constant in their effects. For one, the "French" strain ("F" in the tables), we are indebted to Dr. A. W. Sellards, who had brought it to America from West Africa via England. It had been transmitted to monkeys by Mathis, Sellards, and Laigret (3). The other is the "Asibi" strain ("A" in the tables), with which Stokes, Bauer, and Hudson did most of their work. It was originally obtained on the Gold Coast, West Africa, and was sent to us from Lagos, Nigeria, by Dr. Henry Beeuwkes, Director of the West African Yellow Fever Commission of the Rockefeller Foundation.

Sources of the Virus

In each instance the virus studied was preserved in the blood or the liver tissue of *Macacus rhesus* monkeys.

After an animal was inoculated, the rectal temperature was taken twice a day, and when the temperature rose to 40°C. for the first time, a specimen of blood was taken. The animal was anesthetized with ether and the blood was drawn from the heart.

The sick animals were allowed to die or were chloroformed when moribund. When needed as a source of virus, liver tissue was removed at necropsy with precautions for sterility. In all cases where an animal is reported as having died of yellow fever, the gross lesions of this disease were found on necropsy, and characteristic changes were seen in microscopic preparations of tissues, especially those of the liver. In a few instances the presence of tuberculosis or dysentery com-

plicated the picture and made it difficult to determine the onset and course of the yellow fever. Such cases were excluded from consideration in this study.

Subcutaneous or intraperitoneal injections of fresh first-day blood were almost invariably followed by the development of experimental yellow fever, even when small doses were used. As results with material which had been kept less than nine days are of little interest to this study, they have been omitted from the tables presented, except for the two partial titrations of fresh citrated blood of Monkeys 95 and 118 included in Table I. All injections of virus into monkeys in this study were intraperitoneal except in the case of the two titrations, in which they were subcutaneous. We have noticed no difference between the results following these two methods of inoculation.

The liver tissue proved less reliable for inoculation than the first-day blood. This is as would be expected when one considers that the virus diminishes rapidly in the blood after the first day or two of fever, and probably decreases in the liver during the latter days of the disease. The duration of the illness varies in the different cases and the concentration of virus in the liver at death probably varies also. Whatever the explanation, the liver tissue, though usually infectious, proved to be less consistently so than the first-day blood specimens.

Except when specially noted in the tables, no specimens which failed to infect are included unless the original fresh material was proven infectious by the inoculation of some other specimen derived from it. In a very few cases the inoculated monkeys had previously received material related to yellow fever, most often human blood two or three weeks old, and had shown no reaction. On account of the remote possibility that the animals had been protected by this material the letter "U," for "used," is placed after the number of each of these monkeys in the tables. The weights of monkeys are omitted from the tables, as moderate fluctuations seemed to play no noticeable part in determining the results of the tests. Specimens of monkey blood sent us from Lagos, Nigeria, are included in the tables and identified by specimen numbers preceded by the letter "S" instead of the numbers of the monkeys from which the virus was obtained.

The amounts of blood or liver are stated in the tables in terms of the fresh material. For example, 0.02 gm. of dried blood would be recorded as 0.1 cc., because blood loses about 81 per cent of its weight in drying. 2 cc. of glycerinated blood containing 1 cc. of actual blood would be recorded as 1.0 cc.

Incubation periods are shown in the tables as the interval between inoculation and the first day of fever; for example, if an animal was inoculated in the afternoon and its first fever occurred in the afternoon of the second day after inoculation, the interval would be recorded as 2.0 days, but if the first fever was in the morning of the third day, the period would be 2.5 days. The interval between inoculation and the time of death or recovery is similarly recorded. In the case of recovery the interval is measured to the last recorded temperature of 40°C. or over, and is followed by the letter "R." All deaths recorded were from yellow fever.

In order to limit as far as possible the number of monkeys needed for our investigations, most of the tests of preserved virus for infectivity were made as required by other experiments. This will explain the apparent lack of plan in some of the groups of observations presented.

TABLE I

Preservation of Yellow Fever Virus in Citrated, Clotted, or Glycerinated Blood

Method of preservation	Source, monkey and strain	Amount, blood, fresh	Age of specimen	Monkey inoculated	Interval in days to		Observation period	Result of test inoculation
					Fever	Death or recovery		
		cc.	days	No.			days	
Citration	95 A	0.6	0	99	1.5	5.0		
	95 A	0.08	0	100	2.0	4.5		
	95 A	0.005	0	101	10.0	11.0		
	118 F	0.9	1	129	6.0	7.0 R		Immune
	118 F	0.07	1	117	2.0	4.0		
	118 F	0.006	1	119	—*	8.5		
	18 F	0.6	22	31 U	—	—	24	Not tested
	32 F	3.5	22	29 U	—	—	24	Not tested
	66 F	2.0	30	112	—	—	30	Succumbed
	S 103 A	1.0	35	42	—	—	28	Not tested
	S 104 A	1.0	35	45	3.5	5.5		
	S 105 A	1.0	35	43	—	—	28	Not tested
Clotting	66 F	2.0	30	111	—	—	30	Succumbed
	S 106 A	1.0	35	46	21.0	24.5		
Glycerina- tion	66 F	2.0	30	105 U	6.5	10.5		
	117 F	1.0	60	181	12.0	16.5		
	65 A	1.0	100	179	—	—	29	Succumbed

* No fever was observed before the temperature became subnormal, 6.5 days after inoculation.

Citrated and Clotted Blood

The most convenient method of making direct transfers of yellow fever virus from monkey to monkey was to prevent clotting by the addition of sodium citrate to the freshly drawn blood and to inject the mixture intraperitoneally or subcutaneously. On the day of the bleeding, or even the next day, the virus content of the citrated blood seemed to remain high, as illustrated by the first six items in Table I.

With longer storage the results became increasingly less dependable, and in the six tests of material from 22 to 35 days old there was only one successful inoculation (Table I).

In preparing the citrated specimens, from two to five parts of freshly drawn blood were mixed with one part of sterile 2 per cent sodium citrate solution, usually in 0.9 per cent sodium chloride or Locke's solution. The specimens were then stored in the refrigerator at from 1° to 4°C. The three citrated specimens received from Lagos (S 103, S 104, and S 105 in Table I) consisted of the blood of the same monkey plus an equal quantity of 2 per cent citrate solution. The specimen which proved to be infectious after the long journey from Lagos to New York was one of the two (S 103 and S 104) which had been kept in the ship's refrigerator. The other specimen of the same blood (S 105) had been stored at a higher temperature in the cool room.

If infectious blood is drawn and allowed to clot, it will retain its virulence for about the same length of time as if it had been citrated, as suggested in Table I by the one failure after 30 days in storage and the one success after 35 days. The infectious specimen of clotted blood (S 106) had come from Lagos and was from the same bleeding as the citrated specimens S 103, S 104, and S 105. It had been stored in the ship's cool room. It will be noticed that the incubation period in the case of the successful inoculation with clotted blood was 21 days, the longest in our experience.

Although the virus can be kept in clotted blood about as long as in citrated blood, citration is the more convenient method, for it is necessary to grind the clot with sand and physiological salt solution in a mortar before inoculation. If there is a sufficient amount of serum, this can be used without the clot.

In our experience it appears to be the exception for stored citrated or clotted yellow fever blood to retain its virulence as long as a month, and the method is obviously unsuited for prolonged storage. We made no experiments to determine whether the virus would persist longer in citrated blood under vaseline or oil, or in clotted blood kept continually frozen.

Glycerinated Blood or Liver

As glycerine had proved useful in the preservation of a number of viruses, we made preparations of blood and liver containing 50 per cent of glycerine and tested them for the presence of active virus after storage in a refrigerator at 2° to 6°C. The results are shown in Tables I and II.

The blood specimens were prepared by adding freshly drawn blood to an equal volume of glycerine in a test tube and shaking vigorously. The mixture was a thick fluid which usually clotted gradually in storage until it was of a firm, jelly-

like consistency and required grinding with sand to bring it into solution in physiological salt solution. The liver preparations were of two kinds. The second specimen of glycerinated liver listed in Table II consisted of pieces of liver tissue

TABLE II

Preservation of Yellow Fever Virus in Frozen, Glycerinated, or Dried Liver

Method of preservation	Source, monkey and strain	Amount, liver, fresh	Age of specimen	Monkey inoculated	Interval in days to		Observation period	Result of test inoculation
					Fever	Death or recovery		
		gm.	days	No.			days	
Freezing	65 A*	1+	12	89	—	—	48	Immune
	40 F	1+	15	66	6.5	9.0		
	18 F	1+	18	41	8.5	9.5 R		Not tested
	32 F	3+	20	40	5.5	8.5		
	97 A	1+	21	132	4.5	7.0		
	66 F	1.0	30	124	5.0	7.5		
	55 A*	1.0	30	106	—	—	30	Not tested
	66 F	1.0	100	191	—	—	30	Not tested
Glycerin-ation	66 F	1.0	30	126	6.0	11.0		
	66 F	1.0	30	125	12.5	15.0 R		Immune
	117 F	1.0	60	187	9.0	12.0 R		Immune
	66 F	1+	100	193	1.5	5.0 R		Not tested
Drying	32 F	1.0	9	37	19.0	22.5		
	32 F	0.1	9	24 U	5.5	8.0		
	32 F	0.01	9	14 U	—	—	30	Not tested
	66 F	0.1	30	127	10.0	14.5		
	32 F	0.1	60	94	—	—	30	Not tested
	117 F‡	1.0	63	168 U	—	—	23	Succumbed
	32 F	1.0	100	146	7.0	8.0†		
	66 F	1.0	100	192	4.0	5.0		
	32 F	1.0	150	190	6.5	8.0		

* No control inoculation was made with the original liver tissue of this monkey to prove it infectious.

† Chloroformed early in the disease to secure material for pathological study.

‡ Tubed with calcium chloride.

dropped into 50 per cent glycerine in Locke's solution. The other specimens were prepared by grinding liver in an equal volume of Locke's solution, using sand, and then adding to the resulting suspension an equal volume of glycerine.

The results with glycerinated blood were distinctly better than with citrated or clotted blood, as will be seen in the tables. The samples of glycerinated blood

which had been kept 30 and 60 days were shown to contain active virus, but the incubation period for the 60-day specimen was prolonged. The 100-day specimen failed to infect and produced no immunity.

With the liver specimens we obtained infection in every instance, but recovery followed infection from one of the two 30-day specimens and from the 60-day and 100-day specimens.

The virus of yellow fever apparently can be preserved in 50 per cent glycerine for a longer time than in citrated or clotted blood. Infection has even been obtained with glycerinated material after storage for 100 days, but there is a distinct falling off in virulence with storage after 30 days. Stronger concentrations of glycerine have not been tried.

Frozen Liver

The method of preserving the virus in frozen liver proved to be reliable for periods of at least one month (Table II); but the single specimen tested after 100 days failed to infect. Pieces of liver in test tubes were placed in the freezing compartment of an ordinary household electric refrigerator. As the temperature in the compartment was −12°C. the liver tissue was frozen hard.

On one occasion the refrigerating machinery did not function and the temperature in the compartment rose sufficiently to permit the liver tissue to thaw. As a result the specimens then in storage, the first four in Table II and also the seventh, were under refrigeration for two days without being frozen. The freezing method is simple and valuable when machinery is at hand for maintaining a constant temperature low enough to keep the tissues frozen hard.

Dried Blood or Liver

It was not until we tried drying blood or liver under vacuum in the frozen state and storing it in sealed tubes that we were able to preserve the virus of yellow fever for long periods.

The method was suggested to us by Dr. T. M. Rivers, who had found it satisfactory in the preservation of several viruses including vaccine virus and Virus III. What was essentially the same method had been used by Harris and Shackell (4) in 1911 in preserving rabies virus; and Rous (5) reported that in the same year the active virus of a transmissible sarcoma of fowls was stored successfully in dried and powdered tissue by Murphy. More recently the method of drying while in the frozen state was applied to the preservation of bacterial cultures by Swift (6).

Our usual procedure in preserving the yellow fever virus in dried blood commences with the drawing of 15 to 20 cc. of blood from the heart of a monkey under ether anesthesia on the first day of fever. The needle of the syringe is then thrust through several layers of gauze which cover a sterile, cylindrical glass evaporating dish 12 cm. in diameter and 6.5 cm. deep, and the blood is ejected. The thin layer of fluid blood on the bottom of the dish is then quickly frozen by setting the dish in a shallow pan containing alcohol and pieces of solid carbon dioxide.

At least an hour before the bleeding, a desiccator of the Hempel improved type with an internal diameter of about 15 cm. is packed in an ice-salt mixture in a rectangular metal pan. In the groove in the upper part of the desiccator is about 130 cc. of fresh concentrated sulphuric acid to absorb the moisture given off by the blood. In the bottom is enough glycerine to cover a porcelain platform and make a good contact with the evaporating dish.

When the blood in the evaporating dish is thoroughly frozen, the dish is lifted from the alcohol, the liquid is quickly wiped from the bottom, and the dish is placed in the desiccator. The cover of the desiccator is then given at least one complete turn to make sure that a good contact is made. An extremely small quantity of a suitable thick lubricant has been applied to the contact surfaces in advance. The desiccator is then evacuated with an electric air pump until the pressure has fallen to 1 or 2 mm. of mercury. The desiccator in its pan of ice and salt is put into a refrigerator to prevent rapid melting of the ice. From 16 to 20 hours later the vacuum is released and the desiccator is opened. The evaporating dish is taken out, wiped free of glycerine, and placed on a sheet of paper under a small glass-topped frame. The object of the frame is to prevent the distribution, by air currents, of infectious dust which might be inhaled by the operator. Rubber gloves are worn while the dried material is being tubed.

About ten plugged sterile test tubes measuring 12 by 200 mm. are numbered and weighed in advance. They are taken one by one, warmed slightly in the flame to prevent condensation of moisture, partially filled with the flakes of dried blood, flamed at the mouth, replugged, and again weighed. The gain in weight in grams represents the weight of the dried blood, which is approximately one-fifth of the weight of the fresh blood or of its volume in cubic centimeters. In the evaporating dish the dried blood is in the form of a thin, red, brittle wafer, with fissures running through it. It lies loose from the glass and can easily be broken into bits and transferred to the test tubes with forceps and narrow spatula. It is light and porous and can be readily crushed in a mortar to a smooth powder before it is tubed, but this probably increases the risk of accidental infection and ordinarily is of no advantage. After the weighing, the tops of the plugs are cut off with scissors and the remainder pushed down to the surface of the dried blood with a rod. To make certain of complete dryness, granular calcium chloride is poured in above the plugs before the tubes are sealed in the flame of a blast lamp. The tubes are stored in the refrigerator. The use of calcium chloride, although

perhaps advisable, is not necessary, since the oldest specimens tested were put up without it.   The method of putting in the calcium chloride has been described by J. H. Brown (7).

When the dried blood is required for inoculation, the tube is opened by scratching with a file at the middle of the cotton plug and touching with a hot piece of glass or metal.   The plug is carefully removed and a small amount of 2 per cent sodium citrate in physiological salt solution is put in to prevent scattering of dust. The contents are then emptied into a mortar, the adherent particles are scraped out of the tube with a platinum wire, and the material is ground up with additional fluid.   Without the sodium citrate in the physiological salt solution the dissolved blood will clot in the mortar and make injection difficult.

Small quantities of blood, 1.0 cc. or 0.5 cc., are easily dried in a test tube or ampoule.   The dry blood forms a small button which lies loose in the bottom of the container.   The blood may be put up in ampoules with safety and with great simplicity of technique, but this advantage over the use of the evaporating dish and test tube is more than offset by the greater difficulty of getting the material out.

Where solid carbon dioxide ("dry ice") is not available the blood may be frozen in small amounts (1.0 or 0.5 cc.) in a number of test tubes in an ice-salt mixture before desiccation.

To preserve the virus in liver tissue, from 10 to 20 gm. of the fresh tissue is ground with sterile sand in a mortar without added fluid.   The resulting paste is scraped into the glass evaporating dish and the sterile gauze cover is replaced and tied with string.   Gentle tapping of the dish on a table top will complete the even distribution of the material over the bottom of the dish.   The material is then frozen, dried, and sealed in tubes by the same technique as the one used for blood.   As the total weight of the original material is known, the amount of liver tissue in each tube, in terms of fresh liver, is determined by dividing the original weight of the liver in proportion to the final weights of liver and sand in the several tubes.   Inoculations are made with the material as in the case of blood, but physiological salt solution is used instead of citrate solution.   The dried liver is soft, porous, and friable, and has a light greyish yellow color due to the presence of much fat.

Freezing is not absolutely necessary for the successful preservation of blood.

Specimen 157 A (Table III) was prepared in Lagos by the West African Yellow Fever Commission of the Rockefeller Foundation and was brought to us through the courtesy of Dr. Oskar Klotz of the Commission.   Hudson and Klotz had demonstrated that infected monkey blood, desiccated in a vacuum in the presence of sulphuric acid or calcium chloride, at room temperature or in the icebox, remained infective as long as 38 days (8).   In preparing Specimen 157 A the mixed blood of two monkeys was dried in a vacuum over calcium chloride and sealed

TABLE III

Preservation of Yellow Fever Virus in Dried Blood

Method of preservation	Source, monkey and strain	Amount, blood, fresh	Age of specimen	Monkey inoculated	Interval in days to		Observation period	Result of test inoculation
					Fever	Death or recovery		
		cc.	*days*	*No.*			*days*	
Drying	180 A*‡	2.5	12	202	—	—	32	Not tested
	180 A*‡	0.5	18	205	—	—	32	Not tested
	65 A	0.1	13	95	3.5	7.5		
	S 86 A¶	1.0	28	28	7.0	9.5		
	S 87 A¶	1.0	28	29	—	—	30	Not tested
	66 F	0.1	30	118	4.0	6.0		
	55 A	0.1	31	107	6.0	10.0		
	S 157 A¶	6+	32	103	3.5	7.5		
	117 F‡	0.3	42	144 U	—†	4.0		
	117 F‡	0.3	42	143 U	2.5	4.5		
	117 F‡	0.3	42	145 U	4.0	7.5		
	66 F	0.5	60	147 U	3.5	7.5		
	117 F‡	0.5	60	157 U	1.5	3.0		
	65 A	0.1	61	150	3.0	4.5		
	55 A	0.1	78	152	3.0	5.0		
	117 F‡	1.0	86	209	2.0	2.0‖		
	117 F‡	1.0	94	188 U	1.5	3.5		
	65 A	0.1	100	180	3.5	10.0		
	66 F	0.1	100	188	—	—	30	Succumbed
	55 A	0.1	101	176	9.0	11.0		
	55 A	0.01	101	177	—	—	37	Succumbed
	S 157 A§¶	0.1	102	173	3.5	5.0		
	55 A	1.0	109	151 U	3.0	5.5		
	65 A	1.0	127	210	—	—	30	Not tested
	55 A	1.0	154	235	4.0	6.5		
	S 157 A§¶	1.0	155	231	16.5	19.0		

* This specimen, when 6 days old, produced fever in a monkey in ⌐   and death from yellow fever in 3.5 days.

† No fever observed.

‡ Tubed with calcium chlo⁻¹ ˙

§ Re-tubed ʷⁱᵗʰ

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TABLE IV

Groups of Specimens of the Same Material Preserved in Different Ways and for Varying Lengths of Time

Source, monkey and strain	Blood				Liver			
	Method of preservation	Amount, fresh	Age of specimen	Result. Yellow fever?	Method of preservation	Amount, fresh	Age of specimen	Result. Yellow fever?
		cc.	*days*			*gm.*	*days*	
32 F					Freezing	3+	20	+
					Drying	1.0	9	+
						0.1	9	+
						0.01	9	—
						0.1	60	—
						1.0	100	+
						1.0	150	+
55 A	Drying	0.1	31	+				
		0.1	78	+				
		0.1	101	+				
		0.01	101	—				
		1.0	109	+				
		1.0	154	+				
65 A	Glycerine	1.0	100	—				
	Drying	0.1	13	+				
		0.1	61	+				
		0.1	100	+				
		1.0	127	—				
66 F	Citration	2.0	30	—	Freezing	1.0	30	+
	Clotting	2.0	30	—		1.0	100	—
	Glycerine	2.0	30	+	Glycerine	1.0	30	+
	Drying	0.1	30	+		1.0	30	+ R
		0.5	60	+		1+	100	+ R
		0.1	100	—	Drying	0.1	30	+
						1.0	100	+
117 F	Glycerine	1.0	60	+	Glycerine	1.0	60	+ R
	Drying	0.3	42	+	Drying	1.0	63	—
		0.5	60	+				
		1.0	86	+				
		1.0	94	+				

in a glass tube.   It was transported from Africa to America at room temperature. A part was used in a successful inoculation when the material was 32 days old. The remainder was retubed with calcium chloride and was found to be highly virulent when 102 days old (Table III).   It infected also after 155 days, but the incubation period was prolonged.   The dried blood of this specimen consisted of small black glassy scales in striking contrast to the soft porous red pieces of the blood which was frozen before being dried.   Specimens 86 and 87, from Lagos, were also dried without preliminary freezing.   One of the two was found to be infectious when 28 days old (Table III), but the other was inert.

The specimens of blood and liver which had been frozen and dried seldom failed to produce infection.   Two of the three specimens of blood which were tested when 100 days old were found to be virulent when injected in amounts as small as the equivalent of 0.1 cc. of fresh blood.   One of these two failed to infect at 127 days.   The other was tested again when 154 days old, in a dosage equivalent to 1.0 cc. and produced yellow fever after an incubation period of only four days.   Tests after longer periods have not yet been made.

The dried liver gave similar results, but the observations were fewer (Table II). Our oldest specimen (from Monkey 32) failed to infect when 60 days old when a dose equivalent to 0.1 gm. of fresh liver was injected, but infected after 100 days and after 150 days when the equivalent of 1.0 gm. was injected. ⋅Another specimen 100 days old was tested and found to be infectious.

The process of freezing and drying will preserve bacteria as well as the virus of yellow fever, and one would expect to get bacterial infections occasionally from the use of dried liver, since pathogenic organisms frequently invade the liver late in the disease.   Such infections would occur very seldom when dried blood is used.   As the result of inoculation with a specimen of fresh yellow fever liver we lost two monkeys from a streptococcus peritonitis, and inoculation with another specimen, which had been sent us under refrigeration, caused three animals to develop tuberculous peritonitis.

Comparison of Methods

A close comparison of the value of the methods can only be made by submitting the same original material to the several processes. One specimen of fresh blood or liver may contain many times as much virus as another.   We have, therefore, brought together in groups in Table IV the specimens derived from each of several lots of blood or liver.   Each of these specimens appears also in one of the previous tables, where additional data are given.

CONCLUSIONS

1. The virus of yellow fever may be preserved for at least 154 days in the blood or liver tissue of infected monkeys if the material is dried

in a vacuum while in the frozen state and kept in the refrigator in sealed glass containers. A gradual diminution of virulence is noticeable in the older specimens.

2. If infectious blood is dried in a vacuum at room temperature, instead of in the frozen state, and is stored in sealed containers in the refrigerator, the virus may survive as long as 155 days.

3. The virus may be preserved for at least 30 days in liver kept continuously frozen.

4. Storage of blood or liver in 50 per cent glycerine in the refrigerator will usually keep the virus alive for 60 days and may do so for 100 days, but with the injection of the older material there is a marked tendency toward lengthening of the incubation period and increase in the number of recoveries.

5. Yellow fever virus in citrated or clotted blood, when kept in the refrigerator, dies out rapidly.

6. In our experience the most satisfactory method of preserving strains of yellow fever virus in the laboratory consists of freezing and drying blood taken from a monkey on the first day of an attack of experimental yellow fever and storing the dry material in sealed glass tubes in a cold place.

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A COMPARISON OF THE MANNER OF EXCRETION OF NEUTRAL RED AND PHENOL RED BY THE FROG'S KIDNEY*

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Since the beginning of modern physiological experimentation on the function of the kidney, the study of the manner of its excretion of dyes has proved a fruitful method of attack on the problem of the activity of its complex elements. The literature covering the subject matter of these studies has grown to vast proportions, but needs no review at this time as it has been frequently and thoroughly discussed.

Our interest in the manner of excretion of two dyes, phenol red and neutral red, arose in the course of an investigation of the problems of experimental nephritis which we had produced in frogs and which we studied by means of the newer methods devised by physiologists for the examination of their problems. Among these methods is that of perfusion of the kidney.

The perfusion of the amphibian kidney offers many advantages over similar procedure with mammals. Among the first to use the method extensively were the English observers, Bainbridge and his coworkers (1) and more recently Höber and his pupils (2) have published a long series of studies on the normal function of the frog's kidney. Among their many observations they found evidence indicating that the dye cyanol was not excreted by the tubule but by the glomerulus.

Our original problem was the testing of damaged kidneys, and among other methods that of perfusion was used. One of the reagents

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employed was phenol red. As another line of attack, vital staining with neutral red was employed. It soon became evident that the kidney handled the two dyes in different ways and in view of the conflict in opinion of various workers on the subject it was thought worth while to extend our investigation to a more detailed study of the manner in which the two dyes are excreted by the normal kidney. Our findings seem to warrant consideration for themselves as distinct from their bearing on the more specific problem of the abnormal kidney. They are, therefore, presented separately.

Methods

The method of perfusion which we have used is essentially that devised by Barkan, Broemser and Hahn (3) and elaborated by Höber.
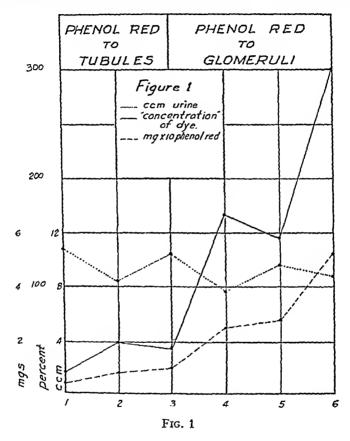
Large bull frogs were used (*R. catesbiana*), averaging 800 gm. in weight, as this allows the collection of considerable amounts of urine, at times as high as 20 cc. or more per hour, and a correspondingly accurate determination of rates of excretion of various substances. The perfusion fluid was that of Barkan, Broemser and Hahn, consisting of a modified Locke's solution buffered to a pH of 7.4 and oxygenated with the proper mixture of CO_2 and O by means of their apparatus. Glycocol in a concentration of .52 per cent was added according to the recommendation of Höber (4) and glucose in a concentration of .05 per cent. The frogs were pithed, the viscera with the exception of the liver and heart, lungs and kidneys removed, with ligation of all severed vessels, and canulae introduced into the anterior abdominal vein—thus supplying the renal portal venous system, —and into the aorta just below the union of the two thoracic branches. In order to restrict the perfusion as much as possible to the kidneys the aorta was then tied below the renal arteries, the large veins to the legs ligated and the vena cava cut to allow the escape of the perfusion fluid which had passed through the kidney. The perfusion pressure was always kept as low as compatible with a free flow of fluid through the vessels, as a rule the venous pressure being 20.0 cm. of water, the arterial 40.0 cm. In both ureters were placed collecting canulae and the urine was removed in 15 minute periods.

Certain routine data were collected in all experiments. The rate of water excretion in cubic centimeters per hour was recorded and the presence or absence of sugar was tested with Benedict's solution. The total electrolyte content was followed by means of a Christiansen ionometer, giving the relative concentration of salts which was expressed as a percentage of NaCl. The concentration of dye in the urine was determined with a Duboscq colorimeter and a known standard. In our findings only one typical example is given, but for each experiment sufficient confirmatory experiments were performed to leave no doubt of the result.

EXPERIMENTAL

The excretion of phenol red when supplied to the tubules alone was compared with its excretion when supplied only to the glomeruli.

In these experiments phenol red in a concentration of 20 mg. per 1000 cc. of perfusion fluid was supplied to the tubules by way of the vein, while Locke's

FIG. 1

solution free of dye was at the same time perfused through the glomerular tufts by way of the aorta and renal arteries. The pressure in the latter was about 45 cc. of water as compared to only 20 cc. in the venous tubular system. Before the addition of the dye the kidneys were perfused as routine with clear Locke's solution through both systems for two 15 minute periods at least to be sure that they were working in a normal manner. This was assumed to be the case, in accordance with Höber's findings (2), when the urine was free of sugar and its salt
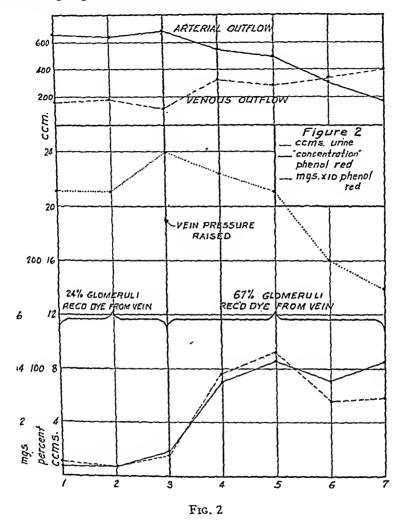
content was not more than 50 per cent of that of the perfusion fluid. After the introduction of the dye, the 15 minute collections were tested in the routine way and the presence of dye noted and its concentration determined. The results are shown in Fig. 1.

During the preliminary periods the urine was sugar free and its salt content 40 per cent of the perfusion fluid. On the introduction of the phenol red into the fluid which went to the tubules no significant change was noted in any of its constituents. The presence of the dye was just perceptible to the eye and on determination was found to be about 50 per cent of that in the perfusion fluid the rate of excretion being .08 mg. per hour. After 3 periods the manner of dye administration was reversed, the concentration previously given to the tubule being led to the glomerular circulation by way of the aorta and renal arteries, while the tubular system of capillaries received clear Locke's solution by way of the renal portal vein. In other experiments dye was added to the glomerular fluid as described above, while the tubular system continued to receive the dye containing Locke's solution with no significant difference in result. The effect of this change in the method of administration of the dye is shown in the 4th period of Fig. 1. The excretion of water, sugar and salt remained practically unaltered but there was a sudden and immediate increase in the dye excretion from a previous level of 50 per cent of the perfusion fluid to 300 per cent. Expressed as rates of excretion, the rise was from .08 mg. per hour to .52 mg. per hour, or a six fold increase. In this particular experiment the increase in the amount of dye excreted by the glomeruli as contrasted to the amount put out by the tubules was not as large as that usually obtained. A summary of nine experiments gave an average increase of twenty-six times, the range of variation being from six to fifty fold.

Evidently phenol red when supplied to the glomeruli of the frog's kidney is excreted at a rate which may be as much as 50 times as great as when it is supplied to the tubules. Some dye, a small amount indeed, is excreted however when the supply is to the tubules alone, and this finding might be interpreted in one of two ways. Either this slight excretion is through that part of the kidney directly supplied by the renal portal system, that is through the tubules, or the fluid from the tubular venous capillaries passes over into the glomerular circulation in small amounts and the dye contained in it is excreted by the glomerulus.

We see no way to devise an experiment that will furnish an absolute control to the first possibility. It is a simple matter, however, to show that the second is not only possible but highly probable, for it has been demonstrated by others that fluid certainly does pass from the tubular to the glomerular circulation, especially if the proper rela-

tions between the pressures in the two systems are not maintained.
The following experiment illustrates this point (Fig. 2).

FIG. 2

After two preliminary periods in which the kidney functioned normally, 20
mg. of phenol red and 2 cc. of India ink suspension were added to 1000 cc. of
Locke's solution in the bottle supplying the venous system, while the arterial bottle
which supplied the glomeruli contained plain Locke's solution. The height of the
two bottles was the usual one, 20 cm. for the venous tubular system and 45 cm. for
the arterial glomerular bed. The out-flow from the two bottles through the two

systems is shown in the graph, the arterial flow being greater than the venous flow. which is the normal relation for perfusion by the method.

As will be seen from the graph, an average concentration of dye equal to only 10 per cent of that of the perfusion fluid was observed, while these conditions prevailed (Periods 1, 2 and 3). This equals a rate of excretion of .04 mg. per hour. The left kidney was then quickly removed with ligation of its vessels so that there would be no leakage during perfusion and fixed in 10 per cent formalin.

The pressure in the venous capillaries was now increased by 10 cm. Thereupon the flow through the venous capillaries increased considerably and though the rate of water excretion remained approximately the same, the concentration of dye rose to 90 and then 110 per cent of that of the perfusion fluid, or a rate of .45 mg. per hour (Periods 4 and 5). This figure is 10 times the rate of the preceding periods. The right kidney was then removed and fixed. Sections of the two kidneys were prepared and examined. The number of glomeruli which contained carbon particles, that is, which had received fluid and dye from the tubular circulation, in the case of the left kidney which had excreted .04 mg. per hour of the phenol red was 24 per cent of the 150 counted. In the right kidney where the rate of dye excretion had risen to .45 mg. per hour, 67 per cent of the glomeruli counted contained carbon as evidence of an increased flow from the tubular venous systems.
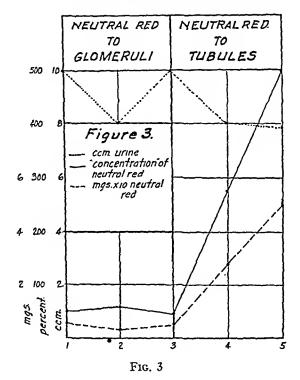
This experiment leaves no doubt that some dye reaches the glomeruli from the venous circulation, that the amount of such dye increases as the pressure on the fluid in the latter system is increased, and that concomitantly with this increased passage of dye to the glomeruli there is an increased rate of dye excretion by the kidney. We therefore believe it likely that the small amount of dye which was observed to be excreted in our first experiment during the period of tubular perfusion was evidence not of tubular excretion of the dye but of an unavoidable imperfection in the method of experiment. Some leakage from the tubular to the glomerular system cannot be avoided so that glomeruli receive the dye, and as the second part of the first experiment shows, it is readily excreted through them.

The excretion of neutral red when supplied to the glomeruli alone was compared with its excretion when supplied only to the tubules.

Before detailing the results of our experiments on the excretion of neutral red by perfused kidneys, it is necessary to test by experiment the statement of certain investigators who claim that neutral red is not excreted by the kidney of the living frog.

10 cc. of saline containing .5 mg. of neutral red was injected into the dorsal lymph sac of a frog whose bladder had been emptied by a catheter. The animal was kept in a glass jar where any urine passed might be recovered. After 60 minutes the bladder was again emptied. 2 cc. of urine was obtained containing .05 mg. of the dye. It follows that 10 per cent of the amount injected was recovered.

The same method of experiment was then applied to rabbits. Five mg. of neutral red was given intravenously in 20 cc. of 3 per cent NaCl solution. After

FIG. 3

1 hour the animal was catheterized again. One rabbit excreted 16 per cent, another 12 per cent of the dye. The average excretion of 8 more animals was 21 per cent with a range from 14 to 31 per cent. As there seems no doubt that neutral red is excreted by both the amphibian and mammalian kidney, the manner of its excretion was further examined by the method of perfusion.

The first perfusion experiment to be described is similar in its method to the first one described with phenol red. Other experiments relating to vital staining of the kidney with the neutral red had led us to suspect that it might be handled in a different manner than is phenol red. For this reason this dye was first introduced into the glomerular system by way of the artery and after a certain number of

periods was then supplied to the tubular apparatus by way of the renal portal vein. Fig. 3 shows the result. During Periods 1, 2 and 3 in which the dye reached the glomeruli in large amount, only small amounts appear in the urine, averaging about 50 per cent of the concentration in the perfusion fluid, an average rate of .06 mg. per hour. The method of administration was then reversed, the neutral red now going direct to the tubules by the vein and the glomeruli receiving clear Locke's from the arterial bottle. The concentration of dye in the urine rose in the next (4th) period to 260 per cent, a rate of .24 mg. per hour and increased in the 5th period to a figure of 500 per cent, 5½ times as concentrated as the perfusion fluid, a rate of .50 mg. per hour.

The rate of excretion of neutral red with tubular perfusion of the dye was therefore 8 times that obtained when the dye was administered to the glomeruli. Summarizing twelve experiments shows that the average increase in the rate of excretion of the neutral red by tubules as contrasted to the glomerular output was thirty-five times, the range of variation being from eight to sixty fold.
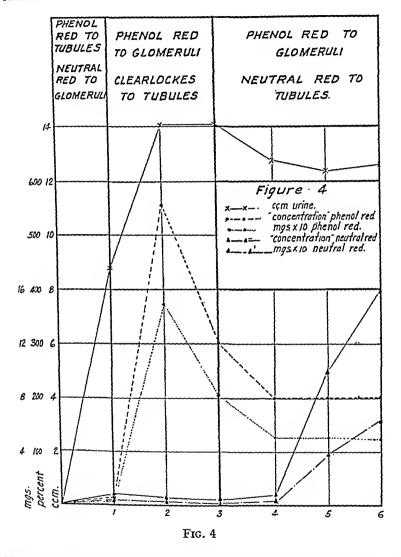
If it be a fact, as the above experiments would seem to indicate that the excretion of the two dyes is by different mechanisms, phenol red being excreted chiefly by the glomerular apparatus and neutral red by the tubular system, then it should be possible to devise crucial experiments which will confirm indirectly the results described above that were obtained by direct observation.

If, for instance, the above hypothesis be true, it should be possible to administer both dyes to kidney at the same time in such a manner that no dye in any considerable amount appears in the urine. The following experiment demonstrates that this can be done.

The perfusion experiments previously described were repeated, but to the glomerular circulation neutral red in a concentration of 12.5 mg. per 1000 cc. was supplied while a similar concentration of phenol red was led to the tubule capillary system. Fig. 4 illustrates the results. Although both perfusion bottles contained highly colored fluid, the urine in the ureteral canulae was practically colorless to the unaided eye. The urine sample for each period was divided into separate portions, one being acidified to produce the red form of neutral red and another alkalinized to show the yellow form of phenol red. In the first sample no phenol red could be demonstrated. Neutral red in an amount averaging 15 per cent of the concentration in the perfusion fluid was present.

Clear Locke's solution, free of either dye, was now supplied to the tubular apparatus by the vein, and phenol red led to the glomeruli by the arteries. In the next period (2) 570 per cent of the concentration in the perfusion fluid appeared

in the urine, a rate of 1.5 mg. per hour.   In the next period about one half as much was excreted.[1]

FIG. 4

Neutral red was now added in its original concentration to the fluid supplying the tubular system, the phenol red still being present in the glomerular supply.

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[1] As we will show in a later study, the dye is excreted in greatest amount in the first period and then falls to a fairly constant level.

By separating the urine as in the first periods, making one acid and the other alkaline, an approximate estimation of the amount of each dye could be made. Exact color matches in the colorimeter were impossible but rough estimations could be made.   In the 5th period the two dyes were present in about equal concentration, 200 per cent for phenol red and 250 per cent for neutral red.   In the 6th period the latter had increased to 400 per cent, the phenol red remaining approximately 200 per cent of the concentration existing in the perfusion fluid.   The final rates of excretion for the two dyes was .5 mg. per hour for neutral red and .45 mg. per hour for phenol red.

It will be seen that the two dyes, when supplied to the kidney the "wrong way to" were not excreted in any significant amount.   When the method of administration was corrected to correspond to the normal manner of excretion, both appeared in urine and were excreted at normal rates.

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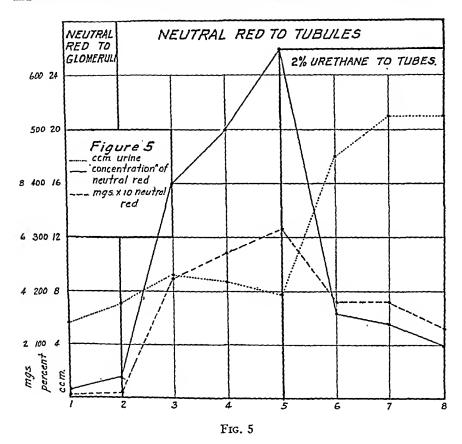
If one of the dyes, neutral red, is excreted by the tubular apparatus, while the other, phenol red, is not excreted in any significant amount by these elements of the kidney but only by the glomeruli, then destroying the function of the tubules should affect the rates of excretion in a very different manner.

Such an experiment involves the complex question of abnormal activity of the tubule epithelium and is one which we shall discuss and study more fully in studies on experimental nephritis in the frog to be published later.   Höber (2) has done a considerable amount of work on this problem in his studies of the physiological activity of the kidney and we can refer to his findings in applying the method to our immediate problem.

Höber has shown that when the tubule cells are anesthetized with urethane, their characteristic activities cease.   There is no longer an absorption of water from the lumen of the tubule, hence a diuresis results.   Sugar, which Wearn and Richards (5) have shown to be present in the glomerular filtrate, is also not absorbed from the urine as it passes down the tubule lumen, and hence appears in the urine, and the absorption of NaCl is also decreased.   The urine, therefore, as a result of the abolition of tubular absorption, approaches in its constitution the character of the perfusion fluid.
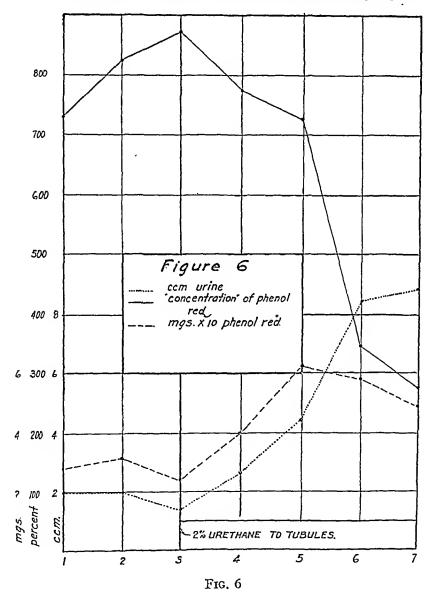
In our experiment the rate of excretion of the two dyes was determined when such conditions had been produced by the action of urethane.   Fig. 5 shows the result of such an experiment.   Neutral red was supplied in the first 2 periods to

the glomeruli alone and, as in the previous experiments, was excreted through them at a very low rate, namely, .02 mg. per hour.   The dye was then led to the tubular system and in three periods (3, 4, 5) an hourly rate of excretion 30 times as high as previously, or .60 mg. per hour, was attained.   The urine during all these periods was normal in every way; the amount was not excessive, sugar was not present and its salt concentration was below that of the perfusion fluid.   Urethane was

FIG. 5

now added in 2 per cent concentration to the perfusion fluid which was passing to the tubules.   There was an immediate drop in the rate of excretion of the neutral red to about one-half its former figure (Period 6) and the rate continued to decrease during the next 2 periods, to a final figure of .22 mg. per hour, or one-third the value found under normal optimum conditions.   All the evidence of tubular damage as described by Höber were present; there was a marked diuresis, sugar appeared in the urine and the salt concentration approached that of the perfusion fluid.

A similar experiment was now performed with phenol red. The results are
shown in Fig. 6. The phenol red was introduced at once in this case into the
glomerular circulation and as before was excreted at the relatively high rate of .3

FIG. 6

mg. per hour. The urine contained no sugar, the salt concentration was only 40
per cent of that of the perfusion fluid and the amount of water was moderate.
The same concentration of urethane, 2 per cent, was then introduced into the fluid
which passed to the tubules. As the chart shows, in Period 6, sugar appeared in

the urine, the rate of water excretion increased 4 fold and the salt concentration rose, all evidences of tubular damage. The rate of excretion of phenol red, however, not only remained undepressed but actually increased.[2] The result is therefore the opposite of that obtained when neutral red was excreted, and gives further evidence which indicates the antithesis in the condition of excretion of the two dyes.

DISCUSSION

Since Nussbaum's original description of the double nature of the blood supply to the amphibian kidney and his experiments, based on this finding concerning the function of the tubules and glomeruli, numerous criticisms have appeared of the basic contention that the blood supply of the two systems is independent. One of the most recent of these is that of Smith (7) who from the result of certain experimental procedures comes to the conclusion that such rich anastomoses occur between the two systems that any interference with either the venous or the arterial systems will cause the unobstructed blood stream to supply both capillary systems. Perfusion experiments as done by many investigators, in so far as the renal portal vein is concerned do not therefore perfuse the kidney in the sense of supplying the tubule through a capillary bed.

A great deal of discussion would be necessary if the details of these experiments of Smith were to be adequately compared with experiments of other authors which are held by them to show the contrary results. For our purpose this can be avoided, for the good reason that our experiments are so designed and their results are of such a nature that in themselves they answer the objections of Smith and others. There is no doubt from our results that the excretion of the dyes is strikingly different when led to the kidney by the arterial and venous routes respectively, and that these differences are the converse of each other with the two dyes. In perfusion of phenol red through the renal portal system for example, the dye did not appear in significant amounts in the urine. This lack of excretion could not be, as Smith's objections might lead one to suppose, because it was passing through

[2] Hayman and Richards (6) have shown that the tubules absorb most dyes, including phenol red, so that abolition of the absorptive function of renal epithelium would increase the amount excreted under such conditions. A more detailed discussion of this point will be given in our studies on experimental nephritis.

the kidney directly to the post caval vein without passing through any capillary bed in the kidney, *because identical perfusion with neutral red through the same system was followed by the excretion of large amounts of dye.* And one can hardly suppose that this excretion of neutral red was the result of fluid reaching the glomeruli by anastomoses from the vein, for the direct introduction of the neutral red into the glomeruli was followed by the excretion of only a small amount.

Since Nussbaum's time there has been no question that anastomosis exists between the two systems. As Höber's experiments have shown (2) leakage from the glomerular system to the tubular is greater than the reverse flow from the venous capillaries to the glomeruli *under normal condition of relative pressures.* With abnormal relations of pressure in vein and artery this leakage of fluid and dye is increased, as our experiments with carbon particles and those of Smith and many others show. Furthermore, in the case of phenol red the apparent excretion of the dye, by the tubules, increases as this leakage increases. It may be true that this output of dye is due to the actual excretion of a small amount through the tubular system into which it is first introduced and that the dye is therefore excreted by both glomeruli and tubules as Tamura and his coworkers (8) have concluded from another type of experiment. But the increase in this apparent tubular excretion coexists with a demonstrable increase of leakage to the glomeruli, through which phenol red is readily excreted, and this fact leads us to believe that the excretion by the tubules is apparent rather than real. The converse we believe to be true of neutral red, the leakage which produces the apparent glomerular excretion of this dye being from the glomeruli to the tubules through the cells of which, as experiment shows, it is readily excreted.

In conclusion we wish to emphasize an important difference in the experiments described here from the majority of the previously reported perfusions of the frog kidney. The high arterial pressure as compared to that used by most investigators, combined with the size of our frogs, often 1000 grams in weight, has allowed us to obtain much larger samples of urine than those usually reported in such experiments. Their volume is, however, not abnormal, as Adolph (9) has shown that the average normal rate of water excretion by the frog when there is an excess of fluid available is 1.3 per cent of their weight per hour. This would mean that with our frogs a perfusion
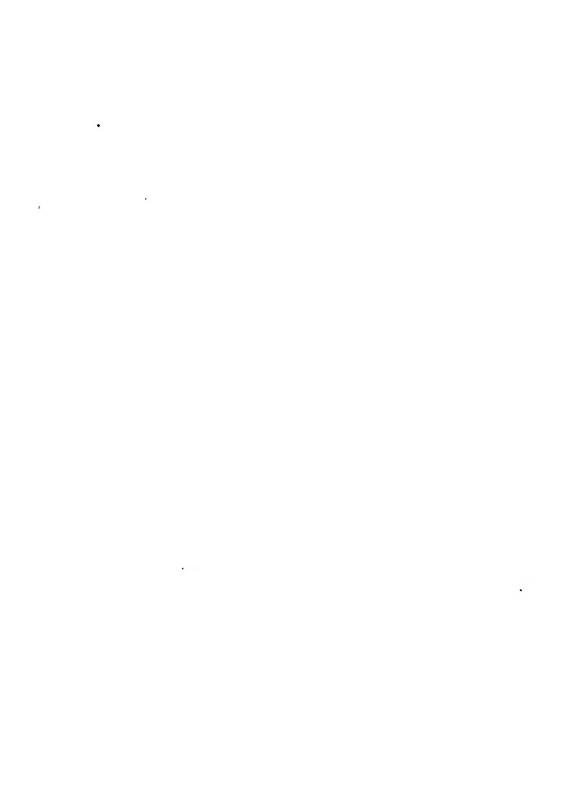
which is normal in its production of water should produce from 7 to 13 cubic centimeters of urine per hour. No objection can be raised to the pressure in the glomerular circulation, which is high as compared to that which exists in the living frog, since it produces no abnormality in the urine. Repeated tests showed that the glomerular membrane was still intact, retaining gum arabic or albumin which had been added to the perfusion fluid, nor did the semi-colloidal neutral red pass through under this pressure, as our experiments have shown. All the other constituents of urine appeared in normal concentration and amount. Under the conditions of our experiments, therefore, the kidneys functioned in a normal manner, a statement which can not be made of experiments where volumes of urine of .19 cubic centimeter were excreted per hour. The added difficulty of accurate quantitative measurements under such conditions is also obvious.

CONCLUSIONS

1. Phenol red and neutral red are excreted by the perfused frog's kidney by different routes.

2. Phenol red is excreted chiefly through the glomerulus, neutral red through the tubules.

3. Some slight excretion of each of these dyes by the converse mechanism is possible, though there is no evidence in our experiments that necessitates such a conclusion.

4. The importance of methods leading to the production of a normal volume of urine by the perfused kidney is emphasized.

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FURTHER NOTES ON THE FILTRATION OF THE VIRUS OF VACCINIA

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In a previous note,[1] attention was drawn to a phenomenon of filtration—first noticed by Bronfenbrenner[2] in connection with bacteriophage—*viz.*, that if tissue containing the viruses of either herpetic encephalitis or vaccinia are suspended in broth instead of saline, filtration of the viruses through diatomaceous filters is greatly facilitated, active filtrates being constantly obtained.

No further work has been carried out with the virus of herpetic encephalitis except to ascertain the minimal lethal dose for a rabbit of the Berkfeld V filtrate. If a 5 per cent emulsion is made in broth of the brain of a rabbit dying of herpetic encephalitis, and the emulsion centrifuged, the minimal lethal dose of the supernatant fluid may be about 0.0008 cc., when injected intracerebrally, whereas the minimal lethal dose of the Berkfeld V filtrate of a supernatant fluid as virulent as this is about 0.06 cc. Thus only about 1 per cent of the virus passes through the filter.

Many filtrations of vaccinia pulp have been carried out since the previous communication, and a very active filtrate was obtained from each lot of material that has been tested, if certain conditions were observed.

Testing of the Filtrate

Every filtrate has been tested carefully for sterility (*i.e.*, absence of visible bacteria), by taking at least 1 cc. of filtrate, inoculating it into hormone broth and observing it during many days of incubation. Since the pulp, as taken from the calf, contains many and various organisms, it was not thought necessary to add

[1] Ward, H. K., and Tang, F.-F., *J. Exp. Med.*, 1929, 49, 1.

[2] Bronfenbrenner, J., *J. Exp. Med.*, 1927, 45, 873.

any test organisms to the material to be filtered. No growth was ever observed in the filtrate growth-control tube in the course of these experiments.

Each filtrate was titrated on the skin of rabbits, and a measure of its activity was thus obtained. Titrations of the same filtrate on different rabbits gave nearly identical results, so that it was felt that a comparison of the titers of different filtrates could be justly made, even if they were not all carried out on the same animals. In carrying out the titrations, the filtrates were diluted 1:4, 1:16, 1:64, 1:256, etc., and 0.2 cc. of the undiluted filtrate and of the successive dilutions were injected intradermally into the shaven skin of normal, well-grown rabbits. Six titrations can easily be carried out on the same rabbit, with three rows of injections on either side of the vertebral column. A No. 27 gauge needle is used, and care taken that the injection is made actually into the skin, and not into the subcutaneous tissue. The reactions are read on the fifth or sixth day after injection. The maximum reaction is an inflamed swelling of firm consistency, about 12 mm. in diameter, sometimes necrotic in the center. As one approaches the endpoint of the activity of the filtrate, the reactions become smaller, the smallest detectable reaction being a pin-point papule. The reactions caused by the filtrate are indistinguishable from those following the injection of the unfiltered emulsion of vaccine pulp.

The intradermal was used in preference to the scratch method in order to obtain more accurate readings.

The Conditions Necessary for Obtaining a Filtrate of Maximum Activity

The technique in use at present will first be described, and then the various factors will be discussed.

Present Technique.—The scrapings from the calf—technically called the pulp— are emulsified and filtered as soon as possible after their removal from the animal. The pulp is weighed roughly, placed in a glass mortar and thoroughly ground with fragments of Pyrex capillary tubing. Enough hormone broth is then added to make a 5 per cent emulsion of the pulp. The broth is added slowly at first, and then in bulk, grinding being continued the whole time in order to obtain as homogeneous an emulsion as possible. The emulsion is then centrifuged at about 2,000 r.p.m. for 30 minutes, and the supernatant poured off. The supernatant fluid is then filtered through a Berkefeld V filter at a negative pressure of about 30 cm. of mercury. The filtrate is pipetted off into tubes and kept in the refrigerator. A small quantity of the filtrate is incubated with hormone broth for some days to test for the presence of bacteria.

The reactions following the intradermal injection into a rabbit of 0.2 cc. of this filtrate and of its successive dilutions, should be as given in Table I.

The Pulp

It was a matter of considerable surprise to find that, if the pulp was kept in the refrigerator for a few days before filtration, the filtrate was barely active, although the unfiltered emulsion had lost none of its activity. Further, the filtrate was inactive, if the pulp was ground up at once and glycerine added before storing in the refrigerator for a few days, and then filtering. Whatever the details of the mechanism of this phenomenon, it was thought likely that the change in filterability was bound up with oxidation of the tissue. And that this appears to be the case is shown by the following experiment:

Fresh pulp was ground up, emulsified in hormone broth and the emulsion centrifuged. The supernatant fluid was then poured off and divided into two parts.

TABLE I

Dilution of filtrate	Reaction	Dilution of filtrate	Reaction
1/1	++++*	1/1,024	+++
1/4	++++	1/4,000	++
1/16	++++	1/16,000	+
1/64	++++	1/64,000	±
1/256	++++	1/256,000	0

* Here and in the following tables, + + + + denotes the maximum reaction, and ± a pin-point papule.

One part was kept in an open tube in the refrigerator; to the other part cystein was added so that a final concentration of 1:1,000 cystein was present, the tube was sealed with vaseline and placed in the refrigerator. This concentration of cystein is ample to prevent oxidation in a closed tube, methylene blue being completely reduced. Both tubes were kept in the refrigerator for 7 days and then filtered through Berkefeld V filters. Both unfiltered emulsions and both filtrates were then titrated. The unfiltered emulsion in the open tube, the unfiltered emulsion in the closed reduced tube, and the filtrate of the latter all proved to be active up to the usual dilution, but the filtrate of the emulsion in the open tube was only very feebly active, giving rise to but pin-point papules in the strongest dilutions. Whether this alteration in filterability in the oxidised tube was due to a lowering of the hydrogen ion concentration and consequent change in the charge on the virus has not yet been ascertained.

The brain of a rabbit infected with neuro-vaccine gave a filtrate of approximately the same activity as the filtrate of calf epidermal lesions.

The Filter.—The filters used in these experiments were the 2½ inch size Berkefeld V filters, autoclaved before using. The majority of them were new filters, but some had been used two or three times. The negative pressure, under which the filtrations were carried out, varied, but usually was about 30 cm. of mercury, although occasionally it was as high as 60 cm., if filtration was slow. The rate of filtration varied with different filters, for reasons which are not understood, but unless filtration was very slow, the rate did not affect the activity of the filtrate. The rate of filtration of course gradually slows, and no more than 50 cc. were ever passed through one filter. Some of the broth filtrates show a slight opalescent haze, but this apparently has no relation to the activity of the filtrate.

TABLE II

Dilution of filtrate	Sand filtrate	Carborundum filtrate	Pyrex glass filtrate
1/1	++++	++++	++++
1/4	++++	++++	++++
1/16	++++	+++	++++
1/64	+++	++	++++
1/256	++	+	++++
1/1,024	+	±	+++
1/4,000	0	0	++
1/16,000	0	0	+
1/64,000	0	0	±

In one experiment a Berkefeld N filter and a Chamberland L1. bis candle were tried. In the case of the Berkefeld N, the undiluted filtrate gave rise to a slight reaction; in the case of the Chamberland, the filtrate was entirely inactive.

The Grinding Material.—In the preliminary experiment sand was used in the glass mortar to help in the grinding of the pulp. Carborundum was tried in place of the sand, but was no improvement. With the idea that perhaps these substances had an absorptive action on the virus, ground up glass was tried. Whatever the reason may be, it was found that Pyrex glass fragments were a definite improvement over sand or carborundum as a grinding material. This is shown in Table II.

To prepare the glass fragments, small pieces of 7 mm. Pyrex tubing are drawn out into short capillaries, which are then cut into 25 mm. lengths and dry sterilized. Before placing the pulp in the mortar, the capillary tubing is ground up roughly, so that, when the pulp is added and grinding commenced, flying fragments of glass do not scatter the pulp over the bench.

The Broth.—The hormone broth is prepared in a somewhat different manner to that originally described by Huntoon.[3]   400 gm. of minced beef heart, 10 gm. of peptone and 5 gm. of salt are boiled for 15 minutes in 1 liter of water.   The meat is then removed by straining through a wire sieve, 24 cc. of N/1 NaOH added, and the broth autoclaved at 15 lbs. for 30 minutes.   The broth is then allowed to stand over night.   Next day the supernatant fluid is syphoned off, adjusted to pH 7.6 and autoclaved at 15 lbs. for 15 minutes.

Meat infusion broth without peptone, a buffered peptone solution, and saline have all been tried, but none of these are as effective a menstruum in which to suspend the pulp as hormone broth.   This is shown in Table III.

TABLE III

Dilution of filtrate	Hormone broth filtrate	Infusion broth filtrate	Peptone filtrate	Saline filtrate
1/1	++++	++++	++++	++++
1/4	++++	++++	++++	++++
1/16	++++	+++	++	++++
1/64	++++	++	+	++
1/256	++++	+	0	+
1/1,024	+++	0	0	0
1/4,000	++	0	0	0
1/16,000	+	0	0	0
1/64,000	0	0	0	0

No further analysis than this has been made of the substance in hormone broth which aids filtration of the virus, but this subject is being investigated by a colleague in the laboratory.

The Properties of the Virus-containing Filtrate

Activity.—As one of the underlying ideas in this work was to obtain a sterile filtrate for use in human vaccination, it was of importance to know in the first place how its activity compares with that of the unfiltered, non-sterile vaccine in general use.   Table IV shows this comparison, which was carried out on the same rabbit on adjacent areas of skin.   This table shows that the unfiltered virus in general

[3] Huntoon, F. M., *J. Infect. Dis.*, 1918, 23, 169.

use for vaccination is about sixteen times more active than the virus filtrate prepared according to the technique described above.

Survival.—In the second place, if the filtrate is to be used as a vaccine, it must be able to survive for a reasonable length of time in the refrigerator. Accordingly, tests were carried out from time to time to see if it was possible to detect any deterioration of specimens kept in the ordinary electric refrigerator. A filtrate prepared and titrated

TABLE IV

Dilution	Vaccinia filtrate	Unfiltered vaccine
1/1	++++	++++
1/4	++++	++++
1/16	++++	++++
1/64	++++	++++
1/256	++++	++++
1/1,024	+++	++++
1/4,000	+++	++++
1/16,000	++	++++
1/64,000	±	+++
1/250,000	0	++
1/1,000,000	0	±

TABLE V

Dilution of filtrate	Filtrate titrated Nov. 6	Filtrate titrated Jan. 22
1/1	++++	++++
1/4	++++	++++
1/16	++++	++++
1/64	+++	++
1/256	++	+
1/1,024	±	0

on Nov. 6 was kept in the refrigerator (about 8°C.) and titrated again on Jan. 22. In this period of 11 weeks, it had deteriorated to about half of its original strength, as is shown in Table V.

Another tube of the same filtrate, kept in contact with the ice-block, but not quite frozen, had hardly deteriorated at all in this length of time, so it is evident that if the filtrate is frozen, it can be kept for long periods of time without any appreciable loss of its activity, and that it only deteriorates slowly at temperatures just above freezing.

As the filtrate loses its activity so slowly in the refrigerator, the influence of glycerine was investigated by keeping the tubes at room temperature. This experiment brought out the fact that in a mixture of equal parts of glycerine and vaccinia filtrate, the virus died out sooner than in control tubes containing vaccinia filtrate alone.

The Nature of the Virus.—Since the virus was suspended in a fluid free from tissue cells and débris, an attempt was made to determine whether it was possible to concentrate the virus by centrifugation.

10 cc. of the filtrate was placed in a conical-ended centrifuge tube and centrifuged at about 2,500 r.p.m. for 4 hours. The supernatant fluid was pipetted off, leaving about 0.1 cc. in the point of the tube. Both the supernatant and the residuary 0.1 cc. were then titrated out. The result is given in Table VI.

TABLE VI

Dilution	Supernatant fluid	Residuary fluid
1/1	++++	++++
1/4	++	++++
1/16	+	++++
1/64	0	++++
1/256	0	+++
1/1,024	0	+

Centrifugation thus causes a definite concentration of the virus at the bottom of the centrifuge tube. A more striking result was obtained with a filtrate which showed a slight haze when examined before centrifugation, and a distinct deposit in the point of the tube after centrifugation, but this result may have been due to the carrying down of the virus by the deposit. Certainly neither the haze nor the deposit was virus alone.

Microscopic examination of neither the whole filtrate, the residuary fluid nor the deposit yielded anything that could be said definitely to be the virus, although suggestive forms were seen in Giemsa preparations and in the dark field.

Complement Fixation.—A careful complement-fixation test was carried out by Mr. Seastone, using a centrifuged concentrate of the vaccinia filtrate as antigen, and an immune calf serum which had a

strong neutralizing action on the filtrate as antibody. The experiment was completely negative.

DISCUSSION

These experiments have demonstrated once again the ease with which a very active sterile vaccinia filtrate can be obtained from vaccinia pulp, if certain conditions are observed. The essential factors in the preparation of such an active filtrate are

1. The use of fresh vaccinia pulp.
2. The grinding of the pulp with Pyrex glass fragments in a glass mortar.
3. The emulsification of this ground-up pulp in hormone broth.
4. Centrifugation of the emulsion and the passage of the supernatant fluid through a Berkefeld V filter.

In the filtration of a virus, assuming that the virus is particulate in nature, there are obviously three fundamental factors governing the passage of the virus through the pores of the filter—the size of the virus; the size of the pores of the filter; and what might be termed its "freeness," meaning by that term its ability to pass through the pores of the filter with no other impediment than that due to its size. Its passage could be prevented either by its adherence to, or retention in larger particles in the fluid to be filtered, or its adherence to the walls of the filter pores. It is probable that hormone broth is more efficient than saline in preventing the adherence of the virus to the walls of the pores, although the actual mechanism of its action is unknown. The glass fragments are probably more efficient than sand in grinding up the virus-containing tissue by reason of their sharp edges, but another reason may be that the virus adheres to the sand, and not to the glass. Another phenomenon which needs further investigation is the action of oxidation in preventing filtration of the virus from an emulsion of the pulp which has been exposed to air for some time, although here again it is probable that a change in the charge on the virus makes it adhere to the pores of the filter.

Although not as active as the unfiltered non-sterile emulsion which is in general use for vaccination against small-pox, a sterile filtrate can be prepared which approaches the former in strength and is probably quite active enough for the purposes of immunization. When

inoculated on to the skin of a calf by the scratch method, the resulting lesions are indistinguishable from those from standard vaccine. A suitable technique for human vaccination has yet to be worked out. Theoretically, there should be more certainty and no danger with intradermal inoculation, as the fluid to be injected contains nothing living but the virus. If the scratch method be used, it would doubtless be necessary to add some substance such as glycerine to make the fluid sticky, as the filtrate alone (consisting mainly of broth) runs off the skin too readily.

Recent work has thrown considerable doubt on the presence in serums which neutralize filtrable viruses *in vivo* of antibodies reacting *in vitro*, and certainly no evidence was obtained in these experiments for the presence of agglutinins, precipitins, or complement-fixing antibodies in a serum which neutralized *in vivo* a filtrate containing the vaccinia virus, using the filtrate as the antigen in the *in vitro* experiments.

Previous differential centrifugation experiments with vaccinia virus have always shown a concentration of the virus in the lower part of the centrifuge tube, but as they have been carried out with an unfiltered emulsion, it could be argued that the virus was simply carried down or contained in the cells and cellular detritus. However, the experiment outlined above—in which a clear filtrate was centrifuged at only a moderate revolution rate—showed a fairly definite concentration of the virus in the lowermost layer. This and the almost complete retention of the virus by a Berkefeld N filter point to it as within the limits of visibility under the microscope. Suggestive forms were seen, but proof of their identity with the virus is still to seek.

It has also been shown in these experiments that at least one filtrable virus can survive perfectly well without the addition of glycerine, if it has been freed from its containing tissue by filtration, and indeed the addition of glycerine seems to be somewhat detrimental to its survival under these conditions.

CONCLUSIONS

1. A very active filtrate can be obtained from vaccinia lesions by grinding up the fresh tissue with glass fragments, emulsifying in hor-

mone broth, centrifuging the emulsion and filtering the supernatant fluid through a Berkefeld V filter.

2. The sterile filtrate so obtained has been shown by comparative titration on rabbits to have about one-sixteenth of the activity of the non-sterile emulsion used in human vaccination.

3. Centrifugation of such a filtrate shows a partial concentration of the virus in the lowermost layer.

4. The virus survives for a long time, if the filtrate is kept near the freezing point, and probably will survive indefinitely if kept frozen. The addition of glycerine is not necessary.

ATTEMPTS TO REPRODUCE RHEUMATIC FEVER IN ANIMALS

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PLATES 1 TO 3

INTRODUCTION

With the assumption that rheumatic fever* is due to some infectious agent, the isolation and identification of the agent has been attempted by bacteriological and immunological methods as well as by animal experimentation. Of the various organisms which have been described in connection with this disease, the streptococcus has held foremost attention and is at present the only one which deserves serious discussion with respect to etiology. An analysis of the present status of our knowledge on the cause of rheumatic disease, with few and practically negligible exceptions, amounts to an evaluation of the possible rôle played by the streptococcus.

The association of the streptococcus with rheumatic disease was first brought into prominence by Poynton and Paine.[1] Whilst many articles have been published on the subject, up to recent years those investigators who had conducted the greater number of animal experiments with streptococci paid little attention to the biochemical characteristics of the organisms with which they were dealing. It is particularly for this reason that it is difficult to analyze and evaluate much of the past literature on this subject.

The first obstacle which presents itself in the acceptance of the streptococcus as the etiologic agent of this disease is that, whereas many investigators claim an extraordinarily high percentage of positive blood cultures (Herry,[2] Freund and

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*We feel that the terms "Acute rheumatic fever, Acute articular rheumatism, Rheumatic pancarditis," etc., are each in themselves too limited to cover descriptively all the possible manifestations of this disease. It would seem that the term "Rheumatic Disease" would be preferable inasmuch as this condition undoubtedly exists during afebrile periods.

Berger,[3] Clawson,[4] Suranyi and Forro[36]), many equally careful investigators have obtained a strikingly small percentage of positive blood cultures (McCrae,[5] Philipp,[6] Cole,[7] Harrison,[8] Swift and Kinsella[9]). These discrepancies, however, may be explained possibly on the basis of different technic employed.

The next objection lies in the variety of streptococci which have been found. Thus, while many investigators have obtained from various sources (though principally blood cultures) the *Streptococcus viridans* (Swift and Kinsella,[9] Freund and Berger,[3] Clawson[4]), some have obtained a non-methemoglobin producing organism (Small,[10] Birkhaug[11]) and some have obtained an occasional hemolytic streptococcus (Clawson,[4] Rosenow[12]). The difficulty is further enhanced by the fact that in the hands of most observers the organisms do not appear to fall into one serological group. The outstanding exceptions to this are: Clawson,[4] who found 7 of his 10 *Streptococcus viridans* strains to fall into one homogeneous serological group, Small,[10] who found that 31 strains of *"Streptococcus cardioarthritidis"* obtained from blood cultures, tonsils, feces, etc., fall into one serological group, and Kreidler,[13] who found that 107 strains of *"Streptococcus cardioarthritidis"* obtained from similar sources also fall into one serological group. It is noteworthy that none of these investigators mention absorption tests to prove that they were not dealing with group agglutination.

The only explanations which would still be consistent with such diametrically opposed findings as regards the streptococcus would be to assume either with Rosenow[12] that the streptococcus need not be of a specific type but with specific affinities, or that in this disease the human tissues respond by a specific reaction to non-type-specific agents, particularly under certain hypersensitive states. The latter hypothesis has been voiced by a number of workers and has recently been stressed by Swift[9] and his coworkers.

Another more outstanding discrepancy lies in the fact that, whereas many have claimed the reproduction by means of streptococci, etc., of one or more of the following lesions: endocarditis, arthritis, chorea, and, more significantly, Aschoff-like bodies (Wassermann, Westphal and Malkoff,[14] Meyer,[15] Poynton and Paine,[1] Shaw,[16] Beattie,[17] Rosenow,[12] Coombs, Miller and Kettle,[18] Jackson,[19] Harzell and Henrici,[20] Henrici,[21] Small,[10] Belk and Jodzis,[22] etc.), others have been able to reproduce similar lesions with a variety of organisms not from rheumatic sources (Cole,[7] Horder,[23] Davis,[24] Thalhimer and Rothschild,[25] Cecil,[26] Topley and Weir[27]).

In connection with the alleged production of Aschoff bodies, it must be pointed out that not only are the published photomicrographs of these lesions in animals entirely unconvincing, as has been pointed

out by some of the last named observers, but, also, that Miller[28] called particular attention to the spontaneous occurrence of inflammatory foci in the myocardium of the rabbit and of the guinea pig and suggested that these lesions may have been mistaken for Aschoff bodies in the past.

The interesting work by Birkhaug[11] which has recently been reported may be mentioned. Birkhaug has obtained a toxin from a non-methemoglobin forming streptococcus which he claims produces a positive skin reaction in a higher percentage of rheumatic patients than in non-rheumatic subjects. Injected into his own wrist joint (after previously sensitizing himself with subcutaneous injections of the toxin), it produced a wandering arthritis resembling rheumatic polyarthritis. No definite conclusions can, however, as yet be drawn from this work. Birkhaug's work on skin reactions has been confirmed by Kaiser,[29] Swift,[9] and his coworkers, and by Edith Irvine-Jones.[30]

Swift and his coworkers, however, showed that rheumatic patients are equally sensitive to filtrates from *Streptococcus viridans*, as well as to vaccines and nucleoproteins. In fact, they hold that rheumatic patients show an intensified reactivity (hyperergy) to these products and suggest the possibility that rheumatic disease may be the expression of some such "hyperergy" produced by a variety of streptococci.

Edith Irvine-Jones[30] found that filtrates made from streptococci of the alpha, beta and gamma variety,[37] whether obtained from throats of normal subjects or from those suffering of rheumatic disease, produced in the latter a higher percentage of positive skin reactions than in normal controls.

It is not our intention to review the literature on rheumatic disease in detail. It is obvious, however, from this brief survey that the subject is still in a chaotic state. For this reason, we thought it advisable to repeat some of the work reported in the past, particularly with the object of reproducing the disease in animals. Moreover, there was to be used a large number of animals of different species and a variety of materials obtained from rheumatic patients (see "experimental" below), including "pedigreed streptococci," *i.e.*, organisms obtained from patients in whom autopsy, biopsy (subcutaneous nodules), or clinical follow-up established the fact that they had suffered of rheumatic disease at the time that the cultures were made. In addition, there was to be employed a variety of procedures calculated to sensitize the animals or render them susceptible to the disease.

EXPERIMENTAL

The material and technique employed in these studies were as follows:

Inocula.—I. Twenty strains of streptococci whose source and cultural characteristics are shown in Table I. In order to indicate that these organisms were obtained from patients suffering of rheumatic fever, they will be referred to briefly as "Rheumatic streptococci." Many of the organisms were passed through animals in order to raise their virulence (see appendix). The procedure adopted was to place the organisms in pure culture with broth in collodion sacs. The sacs were transplanted into the peritoneal cavities in animals of the same species into which the organisms were ultimately to be injected. In the case of rabbits, the strains were passed through the knee joints of successive animals.

II. Apart from the streptococci, there was used other material from rheumatic cases, such as, whole blood, plasma and buffy layer, serum, pericardial and hydrocele fluid, and filtrates from tonsils, subcutaneous nodules, lymph nodes and nasopharyngeal washings. These were obtained from sources indicated in Table I. The lymph nodes, subcutaneous nodules, and tonsils were each ground with sterile sand, extracted with saline and filtered through Berkefeld candles. The filtrates were injected in the manner indicated in the protocols.

Animals.—Seven species of animals were employed, *viz.*, rabbits, guinea pigs, dogs, cats, swine, sheep and calves. Most of the rabbits employed and all of the dogs, cats, and guinea pigs were full grown animals. The sheep were approximately 8 months old. The swine and calves were used shortly after being weaned.

In the several groups of animals employed a variety of procedures were instituted in different combinations.

Portal of Inoculation.—Injections were made intravenously, intratracheally, intracardially, into the tonsils, joints, pericardium, peritoneal cavity and nasopharyngeal mucous membrane.

Physical State of Inoculum.—Washings of 18 hour agar slants, 18 hour cultures in various fluid mediums,* organisms agglutinated in homologous rabbit sera, organisms incubated (sensitized?) in sera from patients suffering of rheumatic disease, organisms mixed with finely divided agar, organisms suspended in 5% gum tragacanth.

Other Procedures.—Infected agar masses were injected subcutaneously into some animals. Large doses of Digitan were injected intravenously into some animals with the object of poisoning the heart muscle and thus lowering its resistance to infection.† In many animals an external jugular or a femoral vein was either

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* Noguchi, Rosenow, dextrose veal infusion broth, dextrose mutton infusion broth, human hormone medium.

† These injections were carried out in rabbits only. Each rabbit received one intravenous injection of 0.5 cc. of Digitan per kilo twice a week to begin with. The dose and number of injections were increased gradually so that after three weeks the animals were receiving 1 cc. per kilo daily.

crushed or painted with 20% silver nitrate. The object of this was to produce a thrombus which might become the seat of infection with the organism subsequently injected intravenously. It was hoped in this way to produce a constant blood stream infection. Many animals were injected intraperitoneally with large doses of saturated Trypan blue in saline (20 cc. per kilo of rabbit injected weekly). The object of this procedure was to attempt a blockade of the reticulo-endothelial system in order to lower resistance to infection. Daily temperature readings were taken but only abnormal temperatures are indicated in the protocols.

For convenience of analysis the experiments will be summarized in three groups, *viz.*, Group A, Group B, Group C. In each group, animals of different species were used with the object of finding a species susceptible to the organism or virus and because it was hoped that the existence of blood vessels in the valves of some of these species (calves, sheep, swine) in fairly high per cent might furnish a means of localizing an infective agent in the valves.

In some of the groups the experimental procedures were relatively simple, hence an abbreviated summary of the technic will be given for these groups. Where the procedures were more complicated, detailed protocols will be given in the appendix.

Group A

Dietrich[31] has claimed that after sensitizing rabbits by means of successive intraperitoneal injections of first, dead organisms (staphylococci) then live organisms, followed after a suitable interval of time by a small intravenous injection of live organisms, he was able to obtain a high per cent of verrucous endocarditis in rabbits. We attempted to repeat this method of sensitization in a variety of species, using streptococci obtained from patients suffering of rheumatic disease, in the hope of producing verrucous endocarditis with, possibly, other rheumatic clinical and pathological manifestations.

Twenty-four rabbits, 12 guinea pigs, 5 dogs, 9 cats, 2 swine, 1 sheep and 2 calves were employed in this experiment. These were divided in three equal subgroups. The first subgroup was injected with Strains 7A-MB and 26A-IS. The second subgroup was injected with Strains 5A-MK, 9A-IS, 3A-EB, 6A-MT, 10A-IS, 4A-RD, and 11A-RG. The third group received Strain 8A-NG. Since, however, there were no conspicuous differences in the results found in these subgroups the procedures detailed in the following pages will refer only to "rheumatic streptococci" without indicating the identity of the individual strains.

TABLE I

No.	Nature of material	Age	Joints	Pericarditis	Nodules	E.C.G.	X-ray	Attacks	Clinical diagnosis
1A-RG	Pos. bld. clt. *Strep. gamma* *	10			4+	Neg.	Pos.††	1	Rheum. heart dis.
2A-JW	Pos. bld. clt. *Strep. mitis* †	7				Neg.	Pos.	2	Rheum. heart dis. Chorea
3A-EB	Pos. bld. clt. *Strep. saliv.*	6		+		?	Pos.	1	Acute rheum. dis. Pericard.
4A-RD	Pos. bld. clt. *Strep. fecal.*	9				?	Pos.	1	Acute rheum. dis.
5A-MK	Pos. bld. clt. *Strep. fecal.*	13	R. elbow, foot				Pos.	2	Acute rheum. heart dis.
6A-MT	Pos. bld. clt. *Strep. gamma*	5			+	?	Neg.	1	Acute rheum. dis.
7A-MB	Pos. bld. clt. *Strep. fecal.*	7	L. hip	+				1	Acute rheum. dis.†°
8A-NG	Pos. bld. clt. *Strep. fecal.*	9	R. knee, foot	+		Pos.**	Pos.	2	Acute rheum. dis. Pericard.
9A-IS	Pos. bld. clt. *Strep. fecal.*	11		+		Neg.	Pos.	4	Acute rheum. dis. Chorea
10A-IS	Pos. bld. clt. *Strep. fecal.*	28	Hips, knees and ankles			?	Pos.	1	Acute rheum. dis.
10B-IS	Buffy layer of cit. bld.								
11A-RG	Pos. bld. clt. *Strep. fecal.*	28	Mult. arthrit.			Neg.	Pos.	2	Acute rheum. dis.
12A-LH	Pos. bld. clt. *Strep. gamma*	45				Pos.		6	Acute rheum. dis. Aur. Fib.
13A-R to 17A-R, Pos. bld. clts., Swift, *Strep. virid.* = his B-39, 38-D, B-38, A-141, A-135, respectively.									
18A-VF	Sterile peric. fld. P. M.	27		+		Pos.		2	Acute rheum. dis. Pericard.
18B-VF	Cit. and defib. bld.								
19A-RB	Cit. and defib. bld.	12		+		Pos.	Pos.	3	Acute rheum. dis. Aur. Fib.
20A-SY	Tonsil filtrate	33				Pos.	Pos.	3	Rheum. dis.
21A-VS	Sterile bld.	9	Mult. arthrit.			Neg.	Neg.	1	Polyarthritis
21B-VS	Tonsil filtrate								
22A-YL	Sterile bld. clt.	19				L.V.P.	Pos.	3	Chr. cardio-valv. dis.
23A-MB	Sterile bld. clt.	32	Mult. arthrit.			L.V.P.		1	Polyarthritis
24A-AW	Sterile bld. clt.	12	Knees, feet			Neg.	Neg.	1	Acute rheum. dis.
25A-RG	Sterile bld. clt.	17	Ankles, wrists	+		?	Pos.	1	Acute rheum. Pericard.

Case no.	Source of material	Titre	Joints	+	+	E.C.G.	X-ray	No.	Diagnosis
26A-IS	Valve clt. *Strep. fecal.*	41				Pos.	Pos.	2	Chr. cardio-valv. dis.‡
27A-SD	Valve clt. *Strep. ignav.*	16		+		Pos.	Pos.	2	Acute rheum. dis. Pericard.†°
28A-FB	Tonsil clt. *Strep. mitis*	10		+	4+	Neg.	Pos.	3	Acute rheum. dis. Pericard.
29A-MM	Sterile peric. fld.	10		+	3+	?		1	Acute rheum. dis. Pericard.†°
29B-MM	Filt. of lymph node	15		+		Pos.	Pos.	2	Acute rheum. dis. Aur. Fib.
30A-MG	Hydrocele fld.	5		+	+	Pos.	Pos.	1	Chr. valv. dis. Ac. Pericard.
31A-AW	Buffy layer of cit. bld.								
31B-AW	Tonsil filtrate								
31C-AW	Serum	20				Pos.	Pos.	1	Acute rheum. dis. Pericard.
32A-AK	Cit. and defib. bld.								
32B-AK	Serum					Pos.	Pos.		
33A-MH	Sterile defib. bld.	35	Shoulders			Pos.	Pos.	6	Chr. cardio-valv. dis. Aur. Fib.
34A-JR	Tonsil filtrate	8				Pos.	Neg.	4	Chr. cardio-valv. dis.
35A-MS	Tonsil filtrate	11		+		Pos.	Pos.	4	Chr. cardio-valv. dis.
36A-IIM	Serum	10		+		L.V.P.	Neg.	3	Ac. rheum. dis. Chr. valv. dis.
37A-JR	Filt. nasophar. wash.	37	Wrists and L. shoulder			Neg.		1	Subacute rheum. arthritis
37B-JR	Cit. and defib. bld.								
38A-MP	Tonsil filtrate	35	Shoulder			Neg.		1	Subacute rheum. arthritis
39A-EZ	Buffy layer of cit. bld.	15		+	+	Pos.	Neg.	3	Chr. cardio-valv. dis.
40A-AL	Filt. of nodule	12			+	?		2	Acute rheum. dis.†°
40B-AL	Filt. of lymph node			+					

*All organisms in this table except 13A-R to 17A-R belong in the gamma group of Brown.[37] **Pos. E.C.G. = finding of either prolonged conduction time, A-V block, S-A block, or auricular fib. †Holman classification.[38] ††Pos, X-ray = mitral or aortic configuration, pericardial effusion, singly or in combination. ‡Confirmed by autopsy. °Aschoff bodies present.

Rabbits

Twenty-four rabbits received from 1 to 6 injections of "rheumatic streptococci." The first intraperitoneal injection consisted of an amount of saline suspension of killed* organisms equivalent to 3 sq. cm. of an 18 hour growth on glucose agar. In the following three intraperitoneal injections, given at 4 day intervals, the number of organisms was doubled each time. The fifth intraperitoneal injection was similar to the first except that living organisms were employed. The sixth injection was given intravenously after a 12 day interval and consisted of one loopful of an 18 hour agar slant of the organisms.

Only six of these rabbits lived long enough to receive an intravenous loopful. These rabbits were exsanguinated under ether anesthesia in from 2 days to 1 month after the last injection. One of the rabbits showed, microscopically, several small scattered areas of accumulations of lymphocytes and mononuclear cells in the heart muscle. Otherwise the gross and microscopic findings in all six rabbits were negative. The remaining 18 rabbits died in from 6 days to 4 weeks after the initial injection. Eleven of the rabbits gave completely negative results. Of the remaining seven, five died with purulent pleurisy and lobular or lobar pneumonia due to organisms other than the streptococci injected. Four showed, microscopically, small scattered areas of scarring in the heart muscle, two showed a low grade pericarditis with mononuclear cells. One showed small focal interstitial collections of lymphocytes in the myocardium. One showed an acute focal interstitial myocarditis. One showed a large, irregular, thrombotic mass on the posterior cusp of the mitral valve. This thrombotic mass contained no demonstrable bacteria. There were no evidences grossly or microscopically of embolic phenomena in any of the organs. It is rather interesting to note that this rabbit received only two intraperitoneal injections of dead, pooled organisms.

Guinea Pigs

Twelve guinea pigs received from 8 to 10 injections of "rheumatic streptococci." The first intraperitoneal injection consisted of an amount of a saline suspension of killed organisms equivalent to 1.5 sq. cm. of an 18 hour growth on glucose agar. In the following three intraperitoneal injections, given at 4 day intervals, the number of organisms was doubled each time. The next three intraperitoneal injections were similar to the first three injections, respectively, except that live organisms were employed. After a lapse of 1 week, the equivalent of one-half a loopful of an agar slant of live organisms was injected intravenously. Six of the guinea pigs received two more intravenous injections of live cultures each equivalent to 500 loopfuls of agar slant, at weekly intervals.

Two guinea pigs died before receiving the intravenous injection. The rest were bled out under anesthesia at intervals varying from 1 day to 4 months after

* Killed by weak phenol solution.

receiving the last injection. None of the animals presented clinical or gross pathological abnormalities. Microscopically, six showed focal interstitial accumulations of lymphocytes and large mononuclear cells in the heart muscle. There was a tendency toward perivascular localization of these inflammatory foci. They were also localized, apparently with predilection, in the auricular muscle at the base of the mitral valve.

Dogs

Five dogs received from 6 to 9 injections of "rheumatic streptococci." The first intraperitoneal injection consisted of an amount of a saline suspension of killed organisms, equivalent to 9 sq. cm. of an 18 hour growth on glucose agar. In the following three intraperitoneal injections given at 4 day intervals, the number of organisms was doubled each time. The next four intraperitoneal injections were similar to the first four injections, respectively except that live organisms were employed. After a lapse of 2 weeks the equivalent of three loopfuls (in 2 dogs, 300 loopfuls) of an agar slant of live organisms was injected intravenously.

The dogs were bled out under anesthesia in intervals of from 2 weeks to 4 months. One dog died of lobar pneumonia before receiving the intravenous injection of live organisms. The other dogs showed no gross or microscopic pathological lesions. Clinically, the dogs had been normal.

Cats

Nine cats received each from 8 to 9 injections of "rheumatic streptococci." The first intraperitoneal injection consisted of an amount of a saline suspension of killed organisms equivalent to 6 sq. cm. of an 18 hour growth on glucose agar. In the following three intraperitoneal injections, given at 4 day intervals the number of organisms was doubled each time. The next four intraperitoneal injections were similar to the first four injections respectively, except that live organisms were employed. After a lapse of 2 weeks the equivalent of two loopfuls (in 1 cat, 1000 loopfuls) of an agar slant of live organisms was injected intravenously. The cats were bled out under anesthesia in intervals of from 1 week to 4 months.

None of the cats showed clinical or gross pathological evidence of disease. One cat showed several interstitial foci of necrosis with lymphocytic infiltration, large mononuclear cells and occasional binucleated cells in the heart muscle (Fig. 1). Four cats showed focal interstitial (in one case, perivascular, Fig. 2) accumulations of lymphocytes and mononuclear cells in the heart muscle. The organs were otherwise normal.

Swine

Two swine received each 9 injections of "rheumatic streptococci." The first intraperitoneal injections consisted of an amount of a saline suspension of killed

organisms equivalent of 12 sq. cm. of an 18 hour growth on glucose agar. In the following three intraperitoneal injections, given at 4 day intervals, the number of organisms was doubled each time. The next four intraperitoneal injections were similar to the first four except that live organisms were employed. After a lapse of 1 week, the equivalent of four loopfuls of an agar slant of live organisms was injected intravenously.

One swine developed slightly swollen knee joints which were neither red nor fluctuating, and a temperature of 104.6° 1 day after receiving the intravenous injection. No cultures were taken from these joints. In several days the joints subsided. 2 weeks after receiving the intravenous injection of organisms, the animal was bled out under anesthesia. All the organs including the joints were negative, macroscopically and microscopically.

The other swine also showed somewhat swollen knee joints the day following the intravenous injection of live organisms. There was no elevation of temperature. These joints were neither red nor fluctuating and subsided in a week. 1 week later the equivalent of 600 loopfuls of an agar slant of live organisms was injected intravenously. 1 month later the animal was bled out under anesthesia. The gross and microscopic findings were negative.

Sheep

One sheep received an intraperitoneal injection of an amount of saline suspension of killed organisms equivalent to 18 sq. cm. of an 18 hour growth on glucose agar. In the following three injections, given at 4 day intervals, the number of organisms was doubled each time. The next four intraperitoneal injections were similar to the first four injections, respectively except that live organisms were employed. Following a lapse of 2 weeks the equivalent of six loopfuls of live organisms was injected intravenously followed in 2 weeks by a similar injection of the equivalent of 600 loopfuls of live organisms. 1 month later the animal was bled out under anesthesia.

Following the third intraperitoneal injection of dead organisms the temperature rose to 107.4°F. 2 days later it fell to normal and continued so until the animal was bled out under anesthesia.

The only positive pathological finding was a few interstitial foci of lymphocytes and mononuclear cells in the heart muscle of the left ventricle.

Calves

Two calves received each 9 injections of "rheumatic streptococci." The procedure and doses were exactly the same as for the sheep in the previous experiment except that 3 weeks after the last intraperitoneal injection of live organisms the calves received each the equivalent of 600 loopfuls of live organisms injected intravenously. 4 months later both calves were bled out under anesthesia. The temperature remained normal throughout the experiment. The findings were the same as those of the sheep in the previous experiment.

Summary of Clinical and Pathological Findings in Group A

Out of the 24 rabbits, 12 guinea pigs, 5 dogs, 9 cats, 2 swine, 1 sheep and 2 calves used in this experiment, marked temperature elevation was noted only in the sheep and in one of the swine. Non-fluctuating arthritis which persisted for a short time only was observed in the swine. Most of the pathological lesions were found in the rabbits as follows: low grade, non-specific inflammatory, myocardial foci in 2, myocardial scarring in 4, purulent pleurisy and lobar or lobular pneumonia in 5, low grade pericarditis in 2, acute focal myocarditis in 1, and a non-bacterial thrombus in the mitral valve of 1. Six guinea pigs and five cats showed low grade, non-specific, inflammatory, myocardial foci. One dog showed a lobar pneumonia.

Group B

In this group 32 rabbits were employed. The object here was to produce in varying combinations thrombosis of a large vein with streptococcal invasion of the thrombus, blockage of the reticuloendothelial system, poisoning of the heart muscle with Digitan and a continuous toxemia (if this were possible) by the injection subcutaneously of 15 cc. agar mass subsequently infected with "rheumatic streptococci."

The technic employed is described under "methods" (see above). The subgrouping of this experiment was as follows:

(a) 4 rabbits received Trypan blue, infected agar mass, thrombosis.
(b) 4 rabbits received Trypan blue, infected agar mass, thrombosis, Digitan.
(c) 4 rabbits received Trypan blue, thrombosis.
(d) 4 rabbits received Trypan blue, thrombosis, Digitan.
(e) 4 rabbits received thrombosis.
(f) 4 rabbits received thrombosis, Digitan.
(g) 4 rabbits received infected agar mass.
(h) 4 rabbits received infected agar mass, Digitan.

Each rabbit received in addition daily intravenous injections of 10 cc. broth culture of live pooled "rheumatic streptococci" consisting of Strains 1A-RG, 2A-JW, 3A-EB, 12A-LH, 26A-IS, 27A-SD, 28A-FB, 13A-R, 14A-R, 15A-R, 16A-R and 17A-R.

It may be said at the outset that there were no special differences in the findings of the various groups. The animals died or were killed in from 1 week to 5 months after the beginning of the experiment. Only seven rabbits survived over 2 months, at about which time they all showed swollen suppurating knee joints from which streptococci were cultured.

All but one of the rabbits had negative blood cultures. In most of the cases,

however, insufficient amounts of blood were obtained for culture because we did not wish to bleed directly from the heart. The positive culture was obtained from a rabbit who had suppurating knee joints and was bled out under anesthesia 3 months after the beginning of the experiments.

Another rabbit with suppurating knee joints was bled out and showed a bacterial (streptococcus) endocarditis involving the entire mitral valve, with acute focal suppurative myocarditis. The myocardium showed numerous streptococcus emboli in the blood vessels. The kidneys showed streptococcus emboli in the arterioles and glomerular loops. There was also a focal embolic glomerulonephritis not quite typical of the Loehlein lesion (Fig. 3). The spleen showed a large amount of brown pigment and an increase in large mononuclear cells.

Apart from this, three rabbits showed focal suppurative nephritis, two showed chronic interstitial nephritis, four showed scattered lymphocytic collections in the myocardium, one showed focal necrosis with calcification in the myocardium, four showed small scattered areas of scarring in the myocardium; one showed a chronic (lymphocytic) interstitial valvulitis of the mitral valve, one showed a suppurative pericarditis.

Group C

For these experiments there were employed 11 swine, 3 calves, and 2 sheep. The procedures in this group were so varied and extensive that it is desirable to record them in abstracted protocols (see appendix). Briefly stated, the animals in this group received numerous injections of agglutinated and unagglutinated "rheumatic streptococci;" of blood clot infected with "rheumatic streptococci;" of possible "virus" material represented by whole blood; plasma; serum; pericardial, pleural, and hydrocele fluid; and filtrates from tonsils, subcutaneous nodules, lymph nodes, and nasopharyngeal washings. This "virus" material was obtained from patients suffering of rheumatic disease (Table I). These materials were introduced through one or more of the following routes: intravenously, intraperitoneally, intracardially, and into the tonsils, trachea, or joints. As will be noted, the procedure was varied somewhat for the different animals.

Summary of Clinical and Pathological Findings in Group C

Swine

The experiments lasted from 1 week to 1 month. The temperature of some of the animals rose at times to 105.7°F., but this could not be correlated with any

of the other findings. None of these animals developed swollen joints or other discernible clinical manifestations. They were all bled out under anesthesia.

One swine (Swine 1) which had received an intracardiac injection, showed at autopsy an adherent pericarditis with acute and subacute inflammatory foci in the pericardium (Fig. 4) as well as in the superficial layers of heart muscle. No bacteria were found in the microscopic sections. Four showed occasional interstitial foci of lymphocytic and mononuclear cells in the heart muscle. Two showed a low grade chronic inflammation of the pleura associated with a fibrinous pericarditis. Two showed a small amount of scarring in the media of the aorta. One showed a mild interstitial valvulitis.

Calves

The three calves used in this experiment died 2 weeks, 5 weeks, and 3 months, respectively, after the beginning of the experiment. The temperature showed no unusual fluctuations. All the injections in these animals were done intravenously.

Two of the calves developed a suppurative arthritis of the knee joints from which streptococci were cultured. One of the calves developed the arthritis 3 weeks after the beginning of the experiment. At autopsy this calf showed an organizing synovitis. The findings were otherwise negative. The other calf (Calf 4) developed arthritis 10 weeks after the beginning of the experiment. At autopsy there was found a small warty vegetation in the left ventricle at about the middle of the interventricular septum (Fig. 5). The vegetation measured 2 x 3 mm., was raised 2 mm. above the endothelial lining, was smooth, with a broad base, and could be scraped off with the finger. Microscopic preparations with hematoxylin and eosin stains showed the vegetation to be covered by endothelium. The bulk of the vegetation showed purplish-pink staining, more or less hyaline material with a scattering of lymphocytes at its base. A few streptococci and Gram positive bacilli were found in the superficial layers of this thrombotic mass. The impression gained was that these organisms were post-mortem invaders. On the other hand, the heart muscle, spleen, and kidney showed many streptococcus emboli in the smaller vessels. A fibrinous pericarditis, a suppurative arthritis and a lobar pneumonia were the other findings in this animal.

The third calf (Calf 5) disclosed at autopsy a lobar pneumonia and a small warty nodule situated on the middle of the right posterior cusp of the aortic valve within the sinus pocket, 2 mm. below the free edge. The thrombotic mass measured 2 x 2 mm., was round and fairly smooth. On section it proved to be a typical streptococcus vegetation. The other organs were negative grossly. Unfortunately, however, the spleen and kidneys were lost before microscopical material was taken.

Sheep

The two sheep employed in this experiment died 9 and 10 weeks, respectively, after receiving the first injection. The temperature remained within normal limits.

One sheep received intravenous injections only. At autopsy there were disclosed lobar pneumonia and, microscopically, a few small interstitial foci of mononuclear cells in the myocardium. The other sheep (Sheep 3) received only intracardiac injections. After the third injection, 9 days after beginning of the experiments, positive blood cultures (streptococci) were obtained. This bacteremia remained until the death of the animal. 2 days before death, small petechiae which appeared to be white-centered were found in the conjunctivae of the animal. At autopsy there was found an organizing non-bacterial, fibrinous pericarditis, massive vegetative endocarditis of the aortic and mitral valves, fresh splenic infarction and bacterial emboli in the spleen, kidneys and heart muscle.

DISCUSSION

We have described a rather extensive attempt to induce rheumatic fever in animals by the use of streptococci isolated from patients suffering of rheumatic disease, as well as by the use of other material which might reasonably be supposed to contain the "virus" of the disease.

By consulting Table I it will be seen that many of the strains of streptococci were obtained from blood cultures in uncontaminated form. An analysis of the older up to the most recent literature on the subject brings out a striking similarity between the cultural characteristics of our organisms and many of those previously reported.

Some of the strains as well as "virus" material, it will be noted, were obtained from patients where a subsequent autopsy confirmed the diagnosis of rheumatic disease. The "virus" material was obtained in all instances from patients in whom the past history, present illness, and subsequent history left little doubt as to the correctness of the diagnosis. We desire to lay especial emphasis on our use of material from cases in which histological examination of biopsy material (subcutaneous nodules) as well as autopsy material made the diagnosis absolutely certain.

In contrast to this, much of the experimental work reported in the literature was carried on with streptococci isolated from contaminated sources, e.g., tonsils, feces, post-mortem material, etc., or blood cultures from patients in whom the diagnosis of rheumatic disease was not confirmed by autopsy. It may, therefore, be argued that the equivocal results obtained by these workers were at least in many instances due to the fact that the etiological agent responsible for the disease (i.e., the specific strain of streptococcus) was not employed.

Scattered references in the literature deal with observations on streptococci as secondary invaders in various conditions. Libman[33] has stressed this point for a number of years. In a recent article on general infections by bacteria, he states:

As I have repeatedly emphasized, we now realize the fact that the anhemolytic streptococci constitute the most important secondary aerobic invaders with which the clinician has to reckon. They can be regarded as almost ubiquitous organisms, having been found in the blood in a great variety of conditions, in lymph nodes of various kinds removed during life, and quite regularly in postmortem examination in organs studied in connection with etiologic investigations of several epidemic diseases.

The questionable value of the employment of streptococci obtained from post-mortem material has been further emphasized by the observations of Epstein and Kugel[32] in this laboratory. These workers, using routine autopsy material from non-rheumatic cases, have obtained streptococci of various types from perfectly normal valves in 40% of their cases, from heart muscle in 47%, from bone marrow in 67%, and from blood cultures in 79% of their cases.

It is obvious, therefore, that the finding of small numbers of such organisms in a vegetation, joint, pericardial cavity, subcutaneous nodule, etc., is by itself slim evidence as to their relation to the disease studied. In the absence of immunological reactions in the patients' blood against these organisms we must rely, at the present time, entirely upon the experimental production of the pathognomonic lesion of rheumatic disease, the Aschoff body, before concluding that the organism is responsible for the disease.

In the evaluation of our work we set for ourselves certain definite criteria which had to be fulfilled in order to establish the production of this disease. These criteria are the reproduction of (1) the Aschoff body, (2) non-bacterial pericarditis, (3) non-bacterial verrucous endocarditis. Of these, we held the Aschoff body to be, par excellence, the desideratum.

The production of migratory arthritis even of a non-bacterial type held for us but passing interest, first because it is not an essential concomitant of the disease in the human, secondly, because such joint conditions can be produced by a variety of agents. It is in the recognition of the experimentally produced Aschoff body that most

of the work in the past has been uncritical. For, whilst it is well known that the Aschoff body in the human heart is somewhat pleomorphic, one is reassured of his diagnosis of this lesion in the human heart even when it occurs in its less characteristic form, by the almost invariable coexistence of verrucous endocarditis, the less frequent occurrence of fibrinous non-bacterial pericarditis, the pathognomonic subcutaneous nodules and the long studied and fairly characteristic clinical course, together with the electro-cardiographic signs. One is further aided by the extensive knowledge of the pathology of the human heart where the tremendous fund of data gathered has shown that there are very few lesions with which the Aschoff body in its most characteristic form can be confused to the critical eye.

This is obviously not the case with experimental animals. Miller[28] has shown that in rabbits and guinea pigs, focal perivascular accumulations of large polyhedral cells with single or multiple nuclei are not infrequently found as spontaneous lesions. This we have been able to confirm. The spontaneous lesions which may occur in other experimental animals are not well known and perhaps it is not superfluous to state that one must guard against confusing such lesions with those intended to be produced experimentally. Further, the rabbit, and possibly other animals, are peculiarly prone to the formation of multinucleated cells in collections somewhat resembling Aschoff bodies following the injection of a variety of toxic substances. Fig. 6 shows such a lesion frequently produced in the rabbit's myocardium by the injection of an organism from the hemorrhagic septicemia group. This illustration can almost be superimposed upon one appearing in a recent publication from Small's laboratory by Belk and Jodzis.[22] These investigators produced this lesion by injections of "*Streptococcus cardio-arthritidis*" in rabbits and make the unequivocal statement that it is an Aschoff body in spite of the fact that they describe the finding of calcium deposition with giant cells in the heart.

Fig. 4 shows foreign body giant cells and polyhedral cells, with red staining protoplasmic granules as shown with the Unna Pappenheim stain, produced in the pericardium of Swine 1 in Group C. No bacteria were found in the preparation. Such a lesion can be easily confused with an Aschoff body to one who is not thoroughly familiar with the protean appearances of the latter.

With our associate, Dr. Benjamin Sacks, we submit the following structure as constituting an Aschoff body. For further details as to localization, Sacks'[34] excellent review may be consulted:

The Aschoff bodies consist essentially of focal collections of characteristic large cells belonging to the family of histiocytes, forming nodules of variable size and shape, those present in the myocardium being located in the interstitial connective tissue, in close relation as a rule to the coronary arterioles, and in the subendocardial tissue not in close proximity to blood vessels. The nodules are rounded, oval, globular, fusiform, spindle-shaped or entirely irregular in outline and save in very exceptional instances are not large enough to be seen with the naked eye. The interstitial nodules develop in the adventitia or peri-adventitial tissue of the coronary arterioles, or at some distance away from the vessel. The nodule may approach the vessel at only one point in its circumference or may spread out in both directions until it surrounds half or even the whole of the circumference. The close proximity of the nodule to the vessel may lead to compression of its lumen, especially when there are two nodules at opposite poles of the circumference. The interstitial connective tissue about the nodules is often edematous and some of the surrounding muscle fibers may undergo degeneration.

The predominating and essential cell of the Aschoff body is a large, polygonal element of variable size and shape, containing one or more nuclei. The cytoplasm, which has an indefinite, often ragged, limiting membrane, is rather homogeneous, non-vacuolated, not distinctly granular and in hematoxylin-eosin sections is distinctly, though not deeply *basophilic*. The nucleus may be ovoid or polymorphous. The former is somewhat vesicular and exhibits a *sharply defined nuclear membrane* with one or more nucleolus-like structures. The polymorphous type of nucleus is generally dark staining, large and occupies a relatively large part of the cell. The cytoplasm when stained by the Unna Pappenheim method with methyl green-pyronin assumes a distinctive brilliant red color, but this tinctorial property is shared by other cells (young cells) to such an extent that it is but rarely, if ever, useful for purposes of identification. The cells do not contain detectable phagocytosed material and no microorganisms or inclusion bodies have thus far been discovered. The multinucleated cells contain two to seven nuclei and in rare instances even more, but the staining properties of these cells are identical with those having a single nucleus. The multinucleated cells differ from the Langhans cells of tuberculosis in the central arrangement of their nuclei and resemble to a certain extent the Dorothy Reed cells of Hodgkin's disease. Their number in the Aschoff nodules is variable; at times they are few and at other times they may comprise as many as half of the characteristic cells. Mitoses have not been encountered. Intermingled with the most peripheral of the large cells and especially at the margins of the nodules there may be a variable number of other types of cells, including polymorphonuclear leucocytes, lymphocytes and plasma cells. At times these cells may be quite numerous.

In the fresher nodules it is common to find the characteristic cells arranged about a central zone of necrotic substance containing little or no fibrin. As the Aschoff bodies grow older, the cells become elongated and lose their characteristic appearance. Fibroblasts grow in among the cells and finally replace them. *Ultimately, the arteriole about which nodules have developed becomes surrounded by an oval or circular band of connective tissue which gives the interstitial tissue in rheumatic infection of the myocardium a very characteristic appearance.* In recurrent or chronic cases, new nodules may develop in such periarterial scars.

No description of the Aschoff bodies is complete without emphasing the extraordinary variability in the number, size, localization and appearance of these lesions, some of these peculiarities already having been referred to. At times the myocardium may be fairly riddled with large nodules accompanied by severe disorganization of the adjacent muscle fibers and what appears to be edema of the entire myocardium. In some cases, sheets of the characteristic cells may be found in localized areas of myocardium or pericardium or invading the valves from the rings. In the endocardium of the left auricle which is often the seat of a gross lesion, the Aschoff bodies are often located in rows about a necrotic collagen or elastic tissue fiber, the nuclei being perpendicular to the fibers or they may be located more deeply in the endocardium or subendocardium in the neighborhood of an irregular area of necrotic tissue, in both cases at some distance away from arterioles.

For the identification of Aschoff bodies, chief emphasis should be laid upon the presence of these nodules in the periarterial tissue of the myocardium with the subsequent development of circular perivascular fibrosis, the tendency for the component cells to arrange themselves about necrotic material (collagen?) and the morphology and tinctorial properties of the characteristic mononuclear and multinucleated cells, especially the basophilic cytoplasm and the dark nucleus with its sharply defined nuclear membrane and nucleoli.

It is precisely because there is here room for individual interpretation that we have insisted on the other two criteria as well, namely, the non-bacterial verrucous endocarditis and the non-bacterial pericarditis.

A number of reports on the experimental reproduction of rheumatic endocarditis describe what is obviously vegetative endocarditis (so-called malignant endocarditis). Apart from the fact that a great variety of organisms can produce this condition (Menzer,[35] Cole,[7] Horder,[23] Thalhimer and Rothschild[55]), we can under no circumstances accept such a lesion as of rheumatic origin.

It might be contended that we are too stringent in our postulates, that possibly species other than human may present an entirely different pathological reaction to the same agent or, indeed, may not

be susceptible to the disease.   While such a state of affairs would be highly regrettable, it does not justify the assumption that vegetative endocarditis is verrucous endocarditis or that focal inflammatory accumulations in an animal's myocardium are the equivalent of Aschoff bodies in the human.   And yet just such assumptions have been made.

Following this preliminary discussion there is little further we need say in the appraisal of our work.   We have found in 24 of our animals focal accumulations of inflammatory cells—at times closely resembling Aschoff bodies and yet so distinct from the latter that we can unconditionally state that we have *not* reproduced the classical Aschoff body. We have also found pericarditis in 8 animals, arthritis in 12 and vegetative endocarditis in 4.   Furthermore, we feel reasonably certain that these lesions were not spontaneous occurrences.   Indeed, in one case (Fig. 5), a lesion was produced in the outflow tract of the left ventricle showing a remarkable resemblance to a rheumatic vegetation. This animal, however, showed embolic bacterial lesions in the kidney and spleen.   In short, whilst many lesions have been produced, we cannot state that we have induced rheumatic disease in animals by the use of streptococci nor by other materials obtained from human cases of this disease; this, despite the fact that we have employed a large number of animals, a variety of species and a variety of procedures far greater than that hitherto reported in the literature.   To this we must add the statement that we have also employed the streptococcus in conjunction with possible "virus" material in the same animals with equally unsuccessful results.

Before drawing final conclusions, we must emphasize that this work does not represent an attempt to disprove the streptococcal theory of the etiology of rheumatic fever.   It is certainly possible that our negative results may have been due to the fact that despite the complexity of our experiments and our deliberate attempts to sensitize our animals to the streptococcus, we failed to induce the necessary receptivity of the tissues, (Swift et al.,[9] Semsroth and Koch[39]) if indeed, such a state of receptivity is an essential prerequisite.

As we have indicated, however, quite positive conclusions have been reached by previous workers.   Inasmuch as we have repeated much of the work reported in the past, and have amplified these experiments

in many ways and on an extensive scale, it seems justifiable to point out that rheumatic fever, recognizable as such, has not been reproduced, and to conclude on the basis of such work that either the streptococcus is not the etiological agent of rheumatic fever, or that the species of animal employed are not susceptible to this disease—at least under the conditions of these experiments—or finally that rheumatic manifestations in the species employed differ markedly from those found in the human. Until it is proved that the last of these assumptions is correct, one is not justified in making it.

CONCLUSIONS

Experiments have been described in which we attempted to reproduce in animals the lesions characteristic of rheumatic fever in the human. A large number of animals representing 7 species was employed. Among other materials, streptococci isolated in pure culture from the blood of rheumatic patients (proved to be so by biopsy or by autopsy) as well as whole blood, plasma, serum, pericardial, pleural and hydrocele fluid, filtrates from tonsils, subcutaneous nodules, lymph nodes, and nasopharyngeal washings obtained from such patients were used in a variety of combinations and with a number of procedures calculated to predispose the animal to the disease.

A discussion is given of the criteria whose fulfillment is essential for the establishment of the experimental production of rheumatic disease in animals.

Judged by these criteria, we have failed to reproduce the disease. This conclusion, we believe, holds true for all the work thus far reported in the literature.

APPENDIX

Protocols of Group C

(The following abbreviations are employed: pld = pooled; IV = intravenously; IP = intraperitoneally; IT = intratracheally; cit. & def. = citrated and defibrinated; NP = nasopharyngeal mucous membrane; agg = agglutinated.)

Swine 1.—(10/30/25) 10 cc. pld Mass 1,* IV. (11/2/25) femoral vein crushed,

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* Mass 1 consists of pooled 1A-RG, 2A-JW, 26A-IS, 27A-SD, 28A-FB.

10 cc. pld Mass 1, IP.   (11/5/25) 10 cc. pld Mass 1, into tonsils.   (11/6/25) 10 cc. pld Mass 1 into tonsils, and 10 cc. IV.   (11/9/25) 10 cc. pld 2A-JW, 21A-VS, 35A-MS, IV.   (11/10/25) blood culture taken (streptococci), 10 cc. pld 2A-JW, 27A-SD, 21A-VS, 35A-MS, IV.   (11/11/25) 10 cc. pld 24A-AW, 28A-FB, 27A-SD, 2A-JW, IT.   (11/17/25),10 cc. pld 1A-RG, 28A-FB, 24A-AW, 26A-IS, 35A-MS, into heart.   (11/18/25) Swine very sick, can scarcely stand.   10 cc. pld 27A-SD, 2A-JW, 22A-YL, into joints.   (11/30/25) Bled out under anesthesia.   (For findings see page 53.)

Swine 4.—(12/9/25) 15 cc. pld 2A-JW, 26A-IS, 3A-EB, 1A-RG, IP. (12/12/25) 15 cc. culture of streptococci recovered from Swine 1, IP.   (12/16/25) 3 cc. pld 3A-EB, 32A-AK, 23A-MB, 33A-HH, IV. Animal died. Organs grossly negative. Auricular muscle lymphocytic interstitial myocarditis.

Swine 5.—(12/8/25) blood culture taken (negative). 5 cc. cit. & def. blood (32A-AK),IV, and 25 cc., IP.   (12/9/25) 10 cc. serum (32B-AK), IV.   (12/16/25) 1 cc. cit. & def. blood (19A-RB), IV, 60 cc., IP.   (12/28/25) 20 cc. cit. & def. blood (18B-VF), IP.   (12/31/25) Blood culture taken (negative.) Bled out under anesthesia. Findings negative.

Swine 6.—(12/20/25) Blood culture (negative). Superficial vein crushed. 10 cc. pld 1A-RG, 2A-JW, 12A-LH, 3A-EB, 26A-IS, IV, 5 cc. into each tonsil, 5 cc. into NP, 1 cc. into each ankle joint, and 25 cc. 23A-MB, IP.   (12/21/25) 10 cc. agar mass infected with pld Mass 1, IP.   (12/22/25) 5 cc. pld Mass 1, IT. (12/23/25) 10 cc. pld Mass 1, IV.   (12/24/25) Bled out under anesthesia. Auricle shows occasional perivascular mononuclear accumulations. Otherwise findings negative.

Swine 7.—(12/20/25) Blood culture (negative).   Large superficial vein crushed. 15 cc. pld Mass 1, IV.   5 cc. into each tonsil and NP, 1 cc. into each of 4 joints. 25 cc. 23A-MB, IP.   (12/21/25) 10 cc. agar mass infected with pld Mass 1, IP. (12/22/25) 5 cc. pld Mass 1, IT.   (12/23/25) 7 cc. pld Mass 1, IV.   (12/28/25) 20 cc. cit. & def. blood (18B-VF), IP.   (12/29/25) 20 cc. pld 12A-LH, 3A-EB, 2A-JW, IV, and 30 cc. IP.   15 cc. agar mass infected with these cultures, IP. 4 cc. post-mortem pericardial fluid (18A-VF) ground up with blood clot of Swine 7, IV. Swine developed edema of lung.   (12/31/25) 17 cc. cit. & def. blood (37B-JR), IP.   (1/2/26) Bled out under anesthesia.   Temperature of this swine rose to 106°F. on 12/26/25.   Only findings are organizing peritonitis.

Swine 8.—(12/20/25) Blood culture (negative).   Large superficial vein crushed. 10 cc. pld Mass 1, IV, 1 cc. into each of 2 joints, 5 cc. into tonsil and NP, 25 cc. 23A-MB, IP.   (12/21/25) 10 cc. agar mass infected with pld Mass 1, IP. (12/22/25) 5 cc. pld Mass 1, IT.   (12/23/25) 10 cc. pld Mass 1, IV.   (12/28/25) 20 cc. cit. & def. blood (18B-VF),IP.   (12/29/25) Swine died. Organizing fibrinous pericarditis and peritonitis. Scattered interstitial collections of lymphocytes and monocytes in pericardium.

Swine 10.—Treatment similar to Swine 8.   (1/7/26) Bled out under anesthesia.   Blood culture (negative).   Fibrinous and mononuclear pericarditis.

Swine 12.—Treatment the same as Swine 8. (1/14/26) Bled out under anesthesia. A few scattered mononuclear interstitial foci. Lymphocytic interstitial valvulitis of mitral valve.

Swine 13.—Treatment the same as Swine 8. (12/29/25) Bled out under anesthesia. Blood culture (negative). Negative findings.

Swine 15.—Treatment the same as Swine 8. (1/18/26) Bled out under anesthesia. Blood culture (negative). Fibrinous pericarditis and pleuritis. Focal lymphocytic and monocytic interstitial collections in myocardium.

Calf 1.—(6/17/26) 50 cc. pld "R" cultures,* IV. (6/18/26) Left femoral vein painted with 20% silver nitrate. 15 cc. ground up blood clot from Calf 2 previously incubated for 24 hours with 1 cc. pld "R" cultures, IV. 40 cc. pld "R" culture, IV. (6/23/26) 50 cc. pld Mass 1 and 2,† IV. (6/24/26) 50 cc. pld Mass 1 and 2, IV. (6/25/26) 10 cc. nasal washings (37A-JR) incubated with 2 cc. pld "R" cultures for 24 hours, IV. 40 cc. Mass 1 and 2 agg with homologous rabbit serum and washed, IV. (6/26/26) 2.5 cc. "R" cultures, 22.5 cc. Mass 1 and 2, and 25 cc. agg Mass 1 and 2, IV. (6/28/26) 30 cc. pld "R" cultures, IV. Repeated for following 2 days. (7/2/26) 20 cc. nasal filtrate (37A-JR) and 40 cc. pld "R" cultures, IV. (7/7/26) 30 cc. hydrocele fluid (30A-MB) and 20 cc. agg "R" cultures, IV. (7/9/26) Blood culture (negative). 20 cc. ground up blood clot from Calf 1 which had been incubated over night with 20 cc. pld Mass 1 and 2, IV. (7/10/26) 20 cc. infected blood clot (as above), 20 cc. hydrocele fluid (30A-MB), and 5 cc. tonsillar filtrates (31B-AW), intravenously. (7/15/26) 24 cc. serum (31C-AW) and 5 cc. "R" cultures, IV. Calf died. Findings completely negative.

Calf 4.—(6/30/26) Collodion sacs containing respectively, 3A-EB, 13A-R, 14A-R, 26A-IS, and pld "R" cultures in broth implanted IP. Left femoral vein painted with 20% silver nitrate, 50 cc. pld "R" cultures, IV. (7/7/26) 50 cc. agg "R" culture, IV. Blood culture taken (negative). (7/10/26) 25 cc. infected blood clot (similar to Calf 1), and 5 cc. tonsil filtrate (31B-AW), IV. (7/15/26) Treatment same as Calf 1. (7/24/26) 10 cc. plasma and buffy layer (31A-AW), and 10 cc. 25A-RG, IV. (7/28/26) 10 cc. filtrates of subcutaneous nodules (40A-AL), 10 cc. filtrates of lymph nodes (40B-AL), 10 cc. pld Mass 1 and 2, and 10 cc. agg Mass 1 and 2, IV. (7/30/26) 10 cc. agg pld "R" cultures and 5 cc. plasma and buffy layer (39A-EZ), IV. (7/31/26) 5 cc. pericardial fluid (29A-MM), 5 cc. tonsil filtrate (34A-JR), and 5 cc. pld "R" cultures, IV. (8/3/26) 10 cc. pericardial fluid (29A-MM), 5 cc. serum (36A-HM), and 5 cc. pld "R" strains, IV. (8/7/26) Right knee joint markedly swollen. (8/19/26) 15 cc. pld "R" cultures, 5 cc. pericardial fluid (29A-MM), 5 cc. lymph node filtrates (29B-MM), IV. (8/26/26) 30 cc. agg Mass 1 and 2, and 5 cc. pericardial fluid

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* "R" cultures consist of 13A-R, 14A-R, 15A-R, 16A-R, 17A-R.

† Mass 2 cultures consist of pooled 2A-EB, 12A-LH, pooled "R" cultures, 2A-JW, and strains recovered from Swine 1.

(29A-MM), IV.   (9/4/26) 40 cc. pld "R" cultures and 10 cc. tonsil filtrate (38A-MP), IV.   Swelling gone from knee joint.   (9/11/26) 30 cc. pld "R" cultures and 10 cc. tonsil filtrate (20A-SY), IV.   (9/17/26) Similar to Sheep 1 on 9/16/26, IV. (9/22/26) Capsules removed from peritoneal cavity and cultured on blood plates and veal broth.   (9/24/26) 50 cc. pld rheumatic capsule strains from Calf 4, IV. (9/25/26) Calf very sick.   (9/27/26) Abdominal wound broke open, dressed. (9/26/26) Calf died.   (For findings see page 53).

Calf 5.—Treatment the same as Calf 4, except that no capsules were implanted IP.   No swollen joints developed.   (8/7/26) Calf died.   (For findings see page 53).

Sheep 1.—(7/29/26) Collodion capsules, containing respectively individual "R" strains, 3A-EB, 26A-IS, organisms obtained from blood of Swine 1, and 2A-JW, implanted IP.   30 cc. pld Mass 1 and 2, IV.   (8/18/26) 20 cc. pld Mass 1, 5 cc. each of pericardial fluid (29A-MM) and lymph nodes filtrate (29B-MM), IV. (8/26/26) 20 cc. agg Mass 1 and 2, and 5 cc. pericardial fluid (29A-MM), IV. (9/4/26) 25 cc. agg Mass 1 and 2, and 5 cc. tonsil filtrate (38A-MP), IV. (9/11/26) 25 cc. agg Mass 1, and 5 cc. tonsil filtrate (20A-SY), IV.   (9/15/26) IP capsules removed and contents cultured on blood slants and mutton broth. (9/17/26) Capsule strains emulsified with 2% agar, 20 cc. injected IV.   (10/2/26) 50 cc. pld capsule strains from Sheep 1, IV.   (10/8/26) Stitches of wound infected. Capsule cultures emulsified with gum Tragacanth (5% emulsion), 25 cc. injected IV.   1 hour later sheep died.   A few interstitial mononuclear foci, otherwise *findings negative.*

Sheep 3.—(10/15/26) 50 cc. agg pld capsule strains from Sheep 1, intracardially. This was repeated on 10/19/26, and on 10/23/26.   (10/25/26) Blood cultures show Gram positive cocci.   (10/26/26) 80 cc. agg pld capsules strains of Sheep 1, and 10 cc. tonsil filtrate (20A-SY), intracardially.   (10/29/26) 80 cc. agg pld capsule strains from Sheep 1, intracardially.   Blood culture (streptococci). (11/4/26) 30 cc. agg pld capsule strains from Sheep 1, intracardially.   (11/9/26) Similar to 11/4/26, but 50 cc. injected.   (11/12/26) Similar to 11/9/26.   Blood culture (Gram positive cocci).   (11/19/29) Blood culture (streptococci). (12/7/26) 5 cc. plasma and buffy layer (10B-IS), 40 cc. agg pld capsule strains from Sheep 1, and 5 cc. 7A-MB, intracardially.   (12/29/26) Sheep died.   (For findings see page 54).

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Area of focal necrosis with lymphocytes and large mononuclear cells in cat's myocardium (Group A).

FIG. 2. Perivascular foci of lymphocytes and large mononuclear cells in cat's myocardium (Group A).

PLATE 2
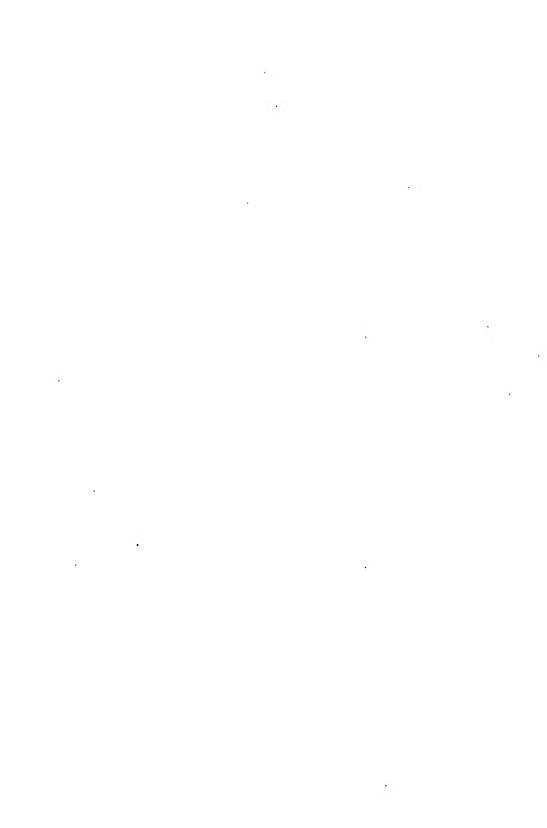
FIG. 3. Focal embolic glomerulo-nephritis in kidney of rabbit (Group B).

FIG. 4. Nodule in pericardium of Swine 1 (Group C), showing collection of large mononuclear cells and giant cells.
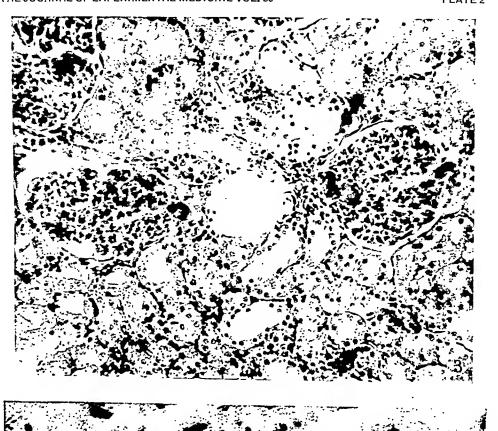
PLATE 3
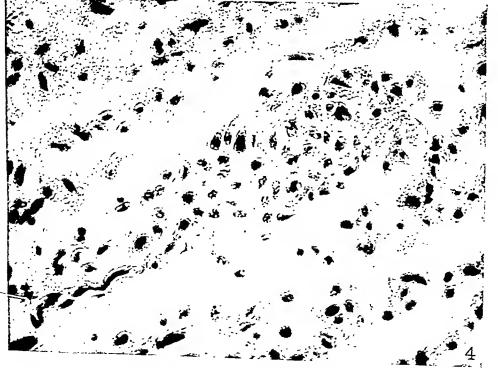
FIG. 5. Verrucous nodule in left outflow tract (interventricular septum) of Calf 4 (Group C).

FIG. 6. Foreign body giant cells and calcium deposit in left auricle of rabbit.

PLATE 1

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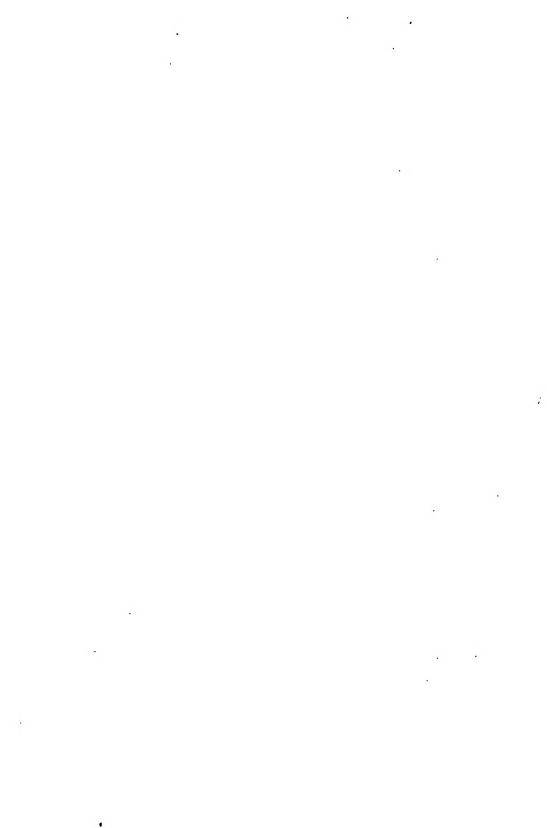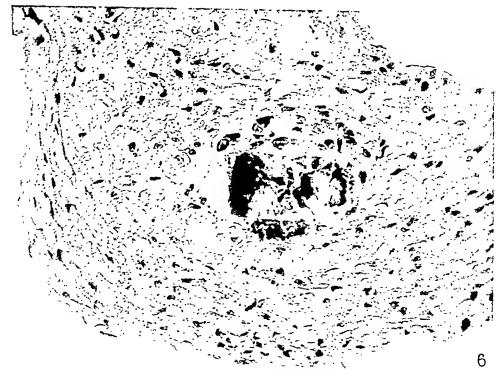
ON THE RELATION OF BACTERIA TO SO-CALLED "CHEMICAL PNEUMONIA"

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PLATES 4 AND 5

I. HISTORICAL

The pneumonia resulting from inhalation of irritating substances has been said to be caused purely by the chemical agents inhaled, and not to be due to the action of bacteria.

Delafield, Prudden and Wood (1) state that "the inhalation of irritating gases, especially chlorine, ammonia, nitrogen tetroxide, bromine, and a large number of the corrosive organic products now used as destructive agents in war, gives'rise to a characteristic type of pneumonia due solely to the chemical action and not necessarily accompanied by bacterial infection, although this may occur."

Wood (2) reports a case of pneumonia occurring from breathing nitric oxide fumes which resulted fatally. The pneumonia was lobular in type, with desquamation of mucous membrane of trachea and bronchi, cellular plugs in the bronchi, and areas of consolidation around the smaller bronchi. The alveoli in the consolidated areas were "filled either with a transparent albuminous mass, or with fibrin containing a few leucocytes, or with desquamated alveolar epithelium, ... Throughout, many alveoli contained red blood corpuscles." Various staining methods showed no organisms. Wood reviewed the literature and found two cases reported by Loeschcke (3), in which a search for bacteria in sections had failed to reveal their presence. Pneumonia induced in animals with nitrogen tetroxide (N_2O_4) fumes was characterized by edema, fibrinous exudate, red and white cells and desquamated alveolar epithelium. Cultures were sterile.

Karsner (4) and Karsner and Ash (5) have shown that rabbits exposed to atmospheres rich in oxygen for 24 to 48 hours, develop a fibrinous broncho-pneumonia in which edema and desquamation of the alveolar epithelium play a prominent part. They think that "the absence of any well marked leucocytic infiltration in the pneumonia area and the absence of demonstrable leucocytosis in the circulating blood point toward a pneumonia of irritative rather than of bacterial origin." However, bacteriological studies of the lungs are not reported.

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Wollstein and Meltzer (6) in the course of some intra-bronchial insufflations found that certain salt solutions caused pneumonia. 2 per cent sodium chloride solution, sodium sulfate, magnesium sulfate, and magnesium chloride solutions produced small patches of broncho-pneumonia. Cultures showed no growth. Mercuric chloride, 1–10,000 dilution, caused hemorrhage, edema and thrombi; no bacteria grew. In another series of experiments (7) they caused broncho-pneumonia by the injection of chloramine-T and Dakin's solution. The pneumonias produced by these solutions were characterized by the absence of fever or appearance of illness. There is no record of bacteriological studies. Kline and Meltzer (8) found that aleuronat and starch caused consolidation resembling that caused by a virulent pneumococcus. Egg yolk and lecithin caused a lobar pneumonia like that caused by a virulent pneumococcus. Cultures were sterile in the majority of their experiments. Wollstein and Meltzer (7) state that "the consolidations of the lung produced by chemical substances differ from infectious pulmonary inflammations only in their sterility."

On the other hand, Winternitz, Lambert, Jackson and Smith (9) find that after chlorine poisoning organisms "that normally inhabit the mouth of the dog find their way into the bronchi and lungs of the dog shortly after gassing and remain there for a long time in animals that survive the acute period." They show "that the pneumococcus and a small Gram-negative hemoglobinophilic organism, normal inhabitants of the dog's mouth, can be cultivated from the lung as early as one-half hour and as late as four days after gassing." Similarly, Winternitz, Lambert and Jackson (10), in studying the bacteriology of pneumonias following gassing with phosgene, found that the bacteriological findings were much the same as in cases of pneumonia in non-gassed dogs.

II. EXPERIMENTAL

In view of the high incidence of pneumonia in the lungs of animals experimentally gassed with toxic compounds in the toxicity work, Medical Research Division, Edgewood Arsenal (11), ample opportunity was offered for the study of the types of pneumonias so caused, and of the incidence of bacteria in these pneumonias.

As cultures of the lungs had not been made at the time of autopsy, the lung sections were stained with Goodpasture-MacCallum stain in order to study the bacteria present. This had the advantage over cultures of giving the number of bacteria present, and showing their position in regard to the lung framework.

The animals studied had been placed in the ice-box as soon as they were found dead, and the autopsy performed as soon as possible thereafter. In studying the sections from these animals the time elapsing between death and autopsy was considered, but this was not found to affect the number of bacteria present when the autopsy was performed within a reasonable limit.

III. METHODS

The tissues were fixed in Zenker-formol solution and embedded in paraffin. The lungs were fixed by running the fixing fluid into the trachea, and then immersing them in the fluid for 24 hours before any sections were taken, as this has been found necessary to preserve the delicate structure and prevent artifacts (12). The sections were stained with hemotoxylin and eosin to study the types of pneumonia, and with Goodpasture-MacCallum stain to study the bacteria present.

IV. DISCUSSION

The types of pneumonia and the bacteria present were studied in the lungs of dogs gassed with mustard gas, methyldichlorarsine, lewisite, phosgene, and some miscellaneous gases.

The significance of the number of bacteria present was considered in reference to:

A. The kind of gas inhaled.
B. The type of pneumonic exudate.
C. The temperature of the animal during illness.
D. The length of time between death and autopsy.
E. The survival after gassing.
F. Bacteria in non-gassed lungs.
   1. In normal lungs.
   2. From pneumonia experimentally caused.
   3. From cases of pneumonia occurring spontaneously in dogs.

A. Relation of Bacteria to the Kind of Gas Inhaled

1. Mustard.—The lungs of mustard gas poisoning show the following lesions in the order of their frequency: hemorrhage; atelectasis; edema; and exudate consisting of epithelial cells, leucocytes, and mononuclear cells, but very little fibrin. In a majority of cases epithelial cells and polymorphonuclear leucocytes are present in large numbers. In a few cases epithelial cells and mononuclears prevail.

Of thirty-two cases studied, nine showed a few bacteria, sixteen showed many and seven a large number. There was no correlation observed between the type of exudate and the number of bacteria.

Table I gives a few typical cases.

2. Methyldichlorarsine.—The pneumonia in methyldichlorarsine poisoning consists of an exudate in which epithelial cells and mononu-

clears predominate. In only a few cases are they out-numbered by polymorphonuclear leucocytes.

In the lungs of eighteen cases, twelve showed few bacteria, two showed many, and four large numbers.

TABLE I

Results Showing Number of Bacteria in a Few Typical Mustard Lungs

General condition of lung	Pneumonic exudate	Bacteria
Hemorrhage Atelectasis	None	Few
Solid with exudate	Leucocytes	Very few
Nearly normal on recovery from gassing	Organized areas and desquamating epithelial cells	Large numbers
Hemorrhage Atelectasis	Leucocytes, epithelial cells	Few
Congestion Hemorrhage	Epithelium, leucocytes	Many
Congestion Hemorrhage Edema	Bronchial exudate	Many
Fibrin, edema	Leucocytes, epithelial cells	Many chains
Necrotic areas	Leucocytes, mononuclears	Enormous numbers in necrotic areas

No correlation was seen between the number of bacteria and the type of exudate, although this gas as a whole showed a small number of bacteria.

Table II gives the summary of findings for methyldichlorarsine in a few typical cases.

3. Lewisite.—A leucocytic exudate prevails in lewisite lungs. In practically all acute deaths leucocytes are predominant with epithelial cells and mononuclears present in much smaller numbers.

Of twenty-seven cases, seventeen showed a few bacteria, three

showed no bacteria, seven had many. No cases showed large numbers of bacteria. The lungs from this series showed fewer bacteria than any other.

4. *Phosgene.*—The first lesion in phosgene poisoning, and the one from which death usually occurs, is edema. If survival is prolonged

TABLE II

Results Showing Number of Bacteria in a Few Typical Methyldichlorarsine Lungs

General condition of lungs	Pneumonic exudate	Bacteria
Congestion Hemorrhage	Scattered leucocytes Epithelial cells Red blood cells	Few
Edema Hemorrhage	Red blood cells Leucocytes	Few, chiefly near bronchi
Hemorrhage Atelectasis Fibrin	Swollen epithelial cells	Few
Edema	Epithelial cells Mononuclears	Very few
Congestion	Leucocytes Few epithelial cells	Few Some intracellular
Fibrin Necrosis Hemorrhage	Leucocytes Few epithelial cells	Necrotic areas loaded with bacteria
Edema	Leucocytes Mononuclears Epithelial cells	Few Some intracellular

the edema fluid becomes infiltrated with leucocytes so that in the later deaths pneumonia is a common finding. The pneumonia is not peri-bronchial in character but is generalized throughout the alveoli as if arising from the alveolar walls. Leucocytes, mononuclears and epithelial cells are present. Most phosgene lungs in which an exudate was present, showed many bacteria.

B. Relation of Bacteria to Type of Exudate

Desquamated epithelium is conspicuous in pneumonias due t
irritating gases but in only a few cases does it form the entire exudat
Usually the picture is that of broncho-pneumonia, with epithelial cell
mononuclears and polymorphonuclear leucocytes composing th
exudate. Frequently the leucocytes out-number the other cells.

The pneumonia of gassed lungs resembles the broncho-pneumoni
seen in non-gassed lungs. Sometimes it is peribronchial in type, some
times it involves a lobule uniformly. Hemorrhage, atelectasis an
perivascular and peribronchial edema are typical findings in gasse
lungs.

A glance at Table III shows that bacteria are prevalent in all type
of pneumonic exudates found in gassed lungs.

TABLE III

Results Showing Relation of Bacteria to Type of Exudate

Type of exudate	Few bacteria	Many bacteria	Large numbers
Leucocytes predominating.................	50%	30%	20%
Epithelial cells predominating..............	33%	47%	20%
Leucocytes and epithelial cells in equal numbers.................................	54%	38%	8%

C. The Temperature of the Animal

As a general rule gassed dogs do not run noticeably high tempera-
tures. This would seem to indicate an absence of bacterial infection,
but the inference cannot be drawn, as no information is at hand as to
what temperature dogs show when infection is known to be present.
A dog's temperature varies so markedly from day to day and from
hour to hour that it does not appear to be an index of the animal's
condition.

The highest temperature seen in the series studied was 106.2°F.
The exudate in this animal was purely leucocytic, but very few bacteria
were found. Another dog with a temperature of 106°F., showed
necrosis of the lungs and large numbers of bacteria. In a dog showing
a temperature of 104.2°F., the exudate was leucocytic and many

bacteria were present, but on the other hand, in several cases showing many bacteria, the temperature was normal.

D. The Length of Time Between Death and Autopsy

There does not appear to be any relation between the time elapsing after death and the number of bacteria.   Some of the cases showing the largest number of bacteria were autopsied very soon after death and some cases showing very few bacteria had been kept as long as 18 hours before autopsy was performed.

TABLE IV

Results Showing Number of Bacteria in Lungs of Dogs Surviving Varying Lengths of Time after Gassing

Hours of survival	No. of cases	Lungs	Bacteria
Less than 12	5	No exudate	Few
12	1	Leucocytes present	Few
12–24	6	Leucocytes and epithelium	Few 4, many 1, some 1
24–36	5	Leucocytes	Few 3, none 2
36–48	4	Leucocytes	Few 3, many 1
48–72	14	Leucocytes	Few 6, many 8
72–96	5	Leucocytes, 2 necrosis	Few 2, many intracellular 3
120	4	Leucocytes, 1 necrosis	Many 4, intracellular
140	5	Leucocytes, 2 necrosis	1 Few, 4 many
9 days	1	Necrosis	Many, intracellular
22 days	1	Leucocytes	Very many
9 weeks	1	Chronic exudate	None

The temperature at which the body was kept after death undoubtedly had more effect on the number of bacteria in the lungs, than did the length of time between death and autopsy.

No record of temperature is available but in view of the well preserved state of the cells it may be assumed that post-mortem degeneration was slight. ·

E. The Survival after Gassing

If the bacteria found in the lungs are present in a causal relationship, it would be expected that the early deaths from pneumonia would

show as many bacteria as the later deaths, or at least that bacteria would be present in considerable numbers in the early deaths. If the presence of the bacteria is merely casual, and due to the injured lungs offering a favorable field for growth, the number might be expected to be smaller in the early deaths and to show an increase as the time of survival is longer.

The cases were grouped and studied therefore with regard to the number of hours of survival (Table IV).

Animals dying in less than 12 hours showed no exudate in the alveoli but a bronchial exudate was frequently present. At 12 hours there was one case of well-developed pneumonia, and above 12 hours most cases showed pneumonia. Areas of necrosis were not observed

TABLE V

Summary of Table IV

Hours of survival	No. of cases	Bacteria
Less than 48 hours	21	None 2, few 16, many 3
48–72 hours	14	Few 6, many 8
72 hours–9 weeks	17	Few 3, many 14, many intra-cellular

in deaths below 72 hours. Above that they were frequent. Above 72 hours many of the bacteria were observed to be engulfed by the leucocytes. Below 72 hours very few intracellular bacteria were seen.

Tables IV and V show that there were more bacteria in the lungs of animals surviving 48 hours or longer, than in those of animals dying at shorter periods of time after gassing.

F. Bacteria in Non-Gassed Lungs

1. In Normal Lungs.—Cultures made from the trachea and bronchi of normal dogs showed plentiful growth. From the lung tissues in most cases there was no growth. Sections of normal lungs stained for bacteria showed a few bacteria on the bronchial mucosa but none in the alveolar structure.

2. In Cases of Experimentally Produced Pneumonia.—Pneumonia

TABLE VI

Results Showing Bacteria Present in Pneumonias Produced by the Inhalation or Injection of Irritating Substances Other than War Gases

Animal	Substance	Method	Death	Condition of lungs	Bacteria	
					Culture	Stain
Dog 5	N_2O_2	Inhalation	Killed, $MgSO_4$	Edema widespread. Exudate of leucocytes, fibrin, red cells	Diplococci	Occasional diplococcus. Few in edema fluid
Rabbit 16	$CuSO_4$ (1% solution)	Intratracheally 1 cc.	Killed	Normal	None	
Rabbit 15	$CuSO_4$ (1% solution)	Intratracheally 1 cc.	Died 11 days	Purulent pleurisy consolidation all lobes	Many colonies	Diplococci
Dog 35-82	$CuSO_4$ (2% solution)	Intratracheally 4 cc.	Died 2 days	Necrosis of trachea, mediastinum and pericardium. Congestion of lungs	Many colonies	Diplococci
Dog 35-83	$CuSO_4$ (2% solution)	Intratracheally 6 cc.	Died 2 days	Edema around trachea and mediastinum and pericardium, necrosis of tracheal mucosa and hemorrhage, edema, consolidation of lungs	Several colonies	Staphylococci, rods
Dog 35-92	$CuSO_4$ (1.5% solution)	Intratracheally 6 cc.	Died 1 day	Edema	No growth. Few colonies from bronchi	Diplococci
Dog 35-93	$CuSO_4$ (1.5% solution)	Intratracheally 5 cc.	Killed 2 days	Congestion, hemorrhage consolidation of lungs	Few colonies	Very minute rods
Dog 35-86	Ethylbrom-acetate	Intravenously	Died 2 days	Hemorrhage, atelectasis, consolidation of lungs	Few colonies	Diplococci

was induced in animals by the inhalation or injection of irritating substances, in order to compare the condition caused with that of gassed lungs. Table VI shows the results. Pneumonia was produced by the inhalation of nitric oxide fumes, by the intratracheal injection

TABLE VII

Results Showing Relation of Bacteria to Exudate in Dogs Dying with Spontaneously Developed Pneumonia

Animal	Death	Condition of lungs	Bacteria	
			Cultures	Stain
Dog 6	Killed, MgSO₄	Consolidation of lungs. Enormous hypertrophy of bronchial mucosa. Bronchial exudate, glands dilated. Alveolar walls thick	Colonies from all lobes	Rods and staphylococci
Dog 7	Died	Consolidation of lungs. Edema. Exudate of epithelial cells, mononuclears, few leucocytes		Staphylococci, few diplococci
Dog 9	Died	Consolidation of lungs. Exudate of leucocytes		Large numbers of bacteria, intracellular
Dog 10	Died	Consolidation all lobes. Exudate of leucocytes and mononuclears		Many bacteria, most of them intracellular
Dog 11	Died	Consolidation all lobes. Exudate of leucocytes, mononuclears and epithelial cells		Many bacteria, intracellular
Dog 12	Died	Consolidation all lobes. Exudate of leucocytes and epithelial cells		Some bacteria, mainly intracellular
Dog 13	Died	Consolidation all lobes. Edema. Exudate of leucocytes, mononuclears and epithelial cells		Many bacteria, intracellular

of copper sulfate solution, and by the intravenous injection of ethyl-bromacetate. None of these pneumonias were sterile.

3. *Pneumonia Occurring Spontaneously in Dogs.*—A number of dogs ng in the kennels were autopsied and found to have consolidation the lungs. These animals had not been gassed at any time. A few were killed with an injection into the heart of concentrated magnesium sulfate solution, when seen to be in bad condition.

The findings in this series are given in Table VII.

In a number of these animals the pneumonic exudate consisted largely of desquamated epithelial cells—the "desquamative pneumonia" of Buhl (13), Fraenkel (14) and Galdi (15), or the "chemical pneumonia" of other authors. That the anatomical picture, which Wood (2) claims is often produced by chemical agents without the aid of bacteria, can be produced by bacteria. alone, is here clearly demonstrated. Furthermore, Galdi (15) has described similar pneumonias in which the infection occurred by the hematogenous route as a sequel to ulcerative endocarditis.

V. CONCLUSIONS

The question of a causal relation of the bacteria in gassed lungs to the pneumonia present cannot be regarded as decided. It may be said that:

1. The appearance of gassed lungs with pneumonia is very similar to the pneumonia of known bacterial origin.

2. In a few cases the type of pneumonia found coincides with the reported cases of so-called "chemical pneumonia," which is characterized by a preponderance of epithelial cells in the exudate.

3. Gassed lungs are not sterile but show highly varying numbers of bacteria.

4. The bacteria are not intracellular and are not present in large numbers in the majority of cases.

The arguments for and against a causal relationship between the bacteria and the pneumonia may be summed up as follows:

1. Against a Causal Relationship

a. The early appearance of pneumonia after gassing.

b. The occurrence of pneumonia with very small numbers of bacteria present.

c. The fact that very few bacteria are engulfed by leucocytes in gassed lungs, whereas large numbers are present in the non-gassed pneumonias and are conspicuously intracellular.

2. In Favor of a Causal Relationship

a. The presence of bacteria in any numbers.

b. The picture of broncho-pneumonia presented is similar to broncho-pneumonia of known bacterial origin.

c. Pneumonias characterized by large numbers of epithelial cells in the exudate (so-called "chemical pneumonia") occur in animals that were never gassed or subjected to other irritating substances in any way.

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EXPLANATION OF PLATES

PLATE 4

FIG. 1. Desquamative pneumonia from lung of dog gassed with phosgene. Symptoms disappeared in 2 days. Killed 1 week later.

FIG. 2. Patch of desquamative pneumonia in a dog that recovered from mustard gassing and was killed 9 months after recovery.

PLATE 5

FIG. 3. Desquamative pneumonia in a dog that was never gassed or exposed to irritating substances of any kind. Killed because it was thought he had worms. Positive culture and bacterial stain from lungs.

FIG. 4. Nitric oxide pneumonia—2 days—leucocytic type.
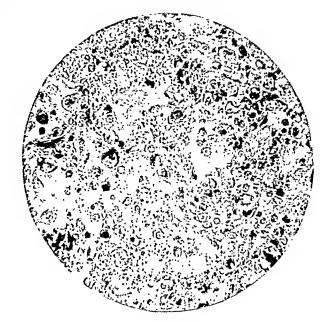
FIG. 1

FIG. 2

FIG. 3

FIG. 4

(Koontz and Allen: Relation of bacteria to "chemical pneumonia")
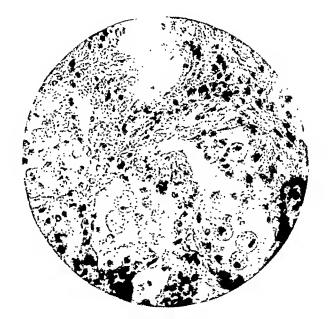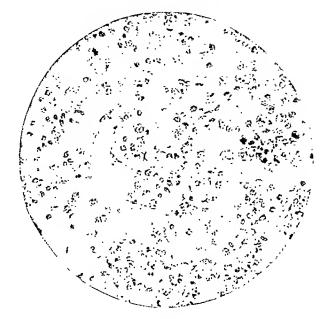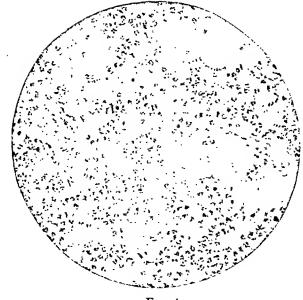
STUDIES ON SOUTH AMERICAN YELLOW FEVER

III. TRANSMISSION OF THE VIRUS TO BRAZILIAN MONKEYS
PRELIMINARY OBSERVATIONS

BY NELSON C. DAVIS, M.D., AND RAYMOND C. SHANNON

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It is our intention to state briefly some of the results obtained in attempts to transmit yellow fever virus to the species of Brazilian monkey popularly known as "prego." This species was used by Noguchi, identified as *Cebus macrocephalus*, and found by him to be susceptible to *Leptospira* infections; the lesions produced closely simulated those of human yellow fever.
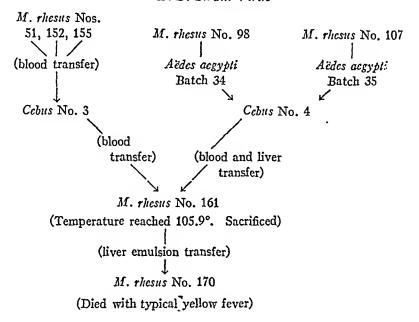
The temperature of all animals inoculated with the virus was taken morning and evening, and in a few instances it rose above 104°F. In several instances the fever appeared early and continued for varying intervals, usually with a tendency to intermissions. It is not improbable that the prolonged fevers may have been due to some other condition than to the inoculation with the virus.

Some animals reacted mildly although the material inoculated was highly virulent for *Macacus rhesus*. Monkeys 6, 9, 12 and 24 fall in this category. In instances in which the inoculum was injected into two animals the effects might vary considerably (Nos. 1 and 2, and 19 and 20).

The virus has been passed from *rhesus* monkeys to *Cebus* and back to *rhesus* by the inoculation of blood and tissues.

On Nov. 16, 1928, *rhesus* No. 161 was given 5 cc. of blood from *Cebus* No. 3 (taken on second day following inoculation); on Nov. 18, 5 cc. of blood from *Cebus* No. 4 (24 hours after mosquito feeding) was inoculated into the same *rhesus* and on the 19th a further transfer of 7 cc. blood-liver mixture was made from *Cebus* No. 4. On Nov. 20 the temperature of No. 161 rose to 105.2°F. and on the 21st it reached 105.9°. The animal was then sacrificed. Liver emulsion from No. 161 was inoculated into *rhesus* No. 170; the latter died and showed typical

Schematic Representation of Transfers Mentioned in the Text

B. B. Strain Virus

M. rhesus Nos.
51, 152, 155   *M. rhesus* No. 98   *M. rhesus* No. 107

(blood transfer)   *Aëdes aegypti*   *Aëdes aegypti*
                                   Batch 34   Batch 35

Cebus No. 3   *Cebus* No. 4

            (blood
                transfer)   (blood and liver
                                    transfer)

M. rhesus No. 161
(Temperature reached 105.9°.   Sacrificed)

(liver emulsion transfer)

M. rhesus No. 170
(Died with typical yellow fever)

Asibi Strain Virus
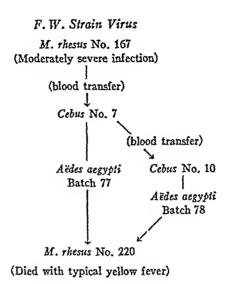
M. rhesus No. 185
(Fatal infection)

(blood transfer)

Cebus No. 11

(blood transfer)

M. rhesus No. 200
(Died with yellow fever)

F. W. Strain Virus

M. *rhesus* No. 167
(Moderately severe infection)
|
(blood transfer)
↓
Cebus No. 7

(blood transfer)

Aëdes aegypti   *Cebus* No. 10
Batch 77

Aëdes aegypti
Batch 78

M. *rhesus* No. 220
(Died with typical yellow fever)

Asibi Strain Virus

M. *rhesus* No. 201
(Fatal infection)
|
Aëdes aegypti
Batch 87
↓
Cebus No. 23
|
(blood transfer)
↓
M. *rhesus* No. 237
(Died with typical yellow fever)

gross and microscopic lesions of yellow fever. On Dec. 10, 1928, *rhesus* No. 200 was injected with blood from *Cebus* No. 11 (taken on the second day after inoculation); again on Dec. 12 No. 200 received blood from the same *Cebus* (taken on the fifth day). No definite fever was detected but on Dec. 14 the temperature fell to 96.4° and the monkey died during the night. The gross picture was typical of yellow fever; microscopically, both liver and kidney showed pronounced necrosis.

The virus has been passed to *Cebus* monkeys by blood inoculation and back to *rhesus* by mosquitoes (*Aëdes aegypti*).

On Dec. 4 *Cebus* No. 7 was inoculated with blood from *rhesus* No. 167, F. W. strain. On the 6th mosquito Batch 77 was allowed to feed and blood was transferred to *Cebus* No. 10. Mosquito Batch 78 was allowed to feed on the latter animal twice (Dec. 7 and 8). On Jan. 2 Batches 77 and 78 fed on normal *rhesus* No. 220. On Jan. 5 this animal registered a temperature of 105.2°F.; fever lasted 3 days and the monkey was found dead Jan. 9. The autopsy lesions were typical of yellow fever. Mosquito Batch 84, fed on *Cebus* No. 12 (B.B. strain), led to a febrile reaction in *rhesus* No. 222, but the animal survived.

The virus has been passed to *Cebus* monkeys by mosquitoes and back to *rhesus* by blood inoculation.

On Jan. 8, 1929, mosquito Batch 87 (infected from *rhesus* No. 201, Asibi strain) was allowed to feed on *Cebus* No. 23. On Jan. 10 this animal had a temperature of 104.2°F., and 1 cc. of blood was transferred to *rhesus* No. 237 by intraperitoneal inoculation. On Jan. 14 No. 237 showed a temperature of 105.6°; on the morning of the 16th the temperature was 96.0°F. and the animal died during the forenoon. Autopsy lesions were typical of yellow fever.

None of the *Cebus* of this series which died or were killed showed definite lesions of yellow fever, either in the gross or microscopically. The work is being continued with the species.

SUMMARY

Yellow fever virus from *M. rhesus* has been inoculated into a South American monkey (*Cebus macrocephalus*) by blood injection and by bites of infected mosquitoes. The *Cebus* does not develop the clinical or pathological signs of yellow fever. Nevertheless, the virus persists in the *Cebus* for a time as shown by the typical symptoms and lesions which develop when the susceptible *M. rhesus* is inoculated from a *Cebus* by direct transfer of blood or by mosquito (*A. aegypti*) transmission.

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IMMUNOLOGICAL STUDIES IN RELATION TO THE SUPRARENAL GLAND

IV. The Effect of Repeated Injections of Epinephrine on the Hemolysin Formation in Suprarenalectomized Rats

By DAVID PERLA, M.D., and J. MARMORSTON-GOTTESMAN, M.D.

(From the Division of Laboratories, Montefiore Hospital, New York)

In previous studies (1)* it was found that epinephrine subcutaneously injected into normal rats in amounts of 0.2 mg. per kilo twice daily during 3 days prior and 4 days subsequent to the injection of 1 cc. of a 10 per cent suspension of sheep cells depressed the antibody forming capacity of normal rats to a marked degree. The average titer was 1/550. In an effort to determine whether the depression of the antibody forming capacity of suprarenalectomized rats (2) is due to a lack of epinephrine the following experiments have been undertaken.

The experiments were divided into two groups. The first group included 30 rats. Of these, 18 were suprarenalectomized, 9 were normal and 3 were rats in which the perisuprarenal fat was torn around both glands. Twelve suprarenalectomized rats were injected subcutaneously with epinephrine in amounts of 0.2 mg. per kilo twice daily from the day of operation during 3 days prior and 4 days subsequent to the injection of 1 cc. of a 10 per cent suspension of sheep cells. Three suprarenalectomized rats received no injections, three suprarenalectomized rats received 0.5 cc. of a 0.9 per cent salt solution twice daily. Of the normal controls, three normal rats received no fluid injections, three received the same amounts of epinephrine as the suprarenalectomized group, three normal rats received physiological salt solution. Three rats in which the perisuprarenal fat was traumatized also received injections of physiological salt solution in the same amounts as the above groups.

In all the experiments recorded, Mulford's "Adrin" (1/1000) was used. In the first group of experiments dilutions were made with a 0.9 per cent salt solution

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* The literature on the effect of epinephrine on antibody formation in normal rats has been reviewed in a previous communication (2).

TABLE I

*Hemolysin Formation After Epinephrine-salt Solution Injections in
Suprarenalectomized Rats*

Rat No.	5th day	8th day	11th day	Weight at opera.	Weight at last titer
				gm.	gm.
370	1600	1000	400	195	187
371	2000	2000		180	180
372	8000			190	180
373	2000	1000	400	180	170
374	600	100		220	192
375	6000	2000		200	197
431	600	160		190	180
432	1000			190	190
433	2000			197	155
434	3000	1000	200	175	175
435	80	200	300	225	180
436	1000	300	600	250	220
Av. titer....	2323	862	380		

Suprarenalectomized Controls

455	400	40	100	160	145
450	200			235	220
453	1000	4000		175	145

Suprarenalectomized Saline Controls

438	160	100	60	175	150
439	12000	1000	200	195	190
440	3000	1000	300	150	120

Normal Controls

427	1600			275	285
428	6000	2000		280	292
501	4000	3000	200	140	145

Normals + Adrin

446	800	100	100	150	155
447	80	30	60	145	150
448	80	1000		135	140

*Normals + Saline**

462	8000	2000	800	215	220
463	16000	12000	6000	205	215

Traumatized + Saline

441	3000	1000	400	180	175
442	3000	1000	300	155	160
443	16000	1000	400	185	175

* Physiological salt solution injected in amounts of 0.5 cc. subcutaneously twice daily during 3 days prior and 4 days subsequent to injection of sheep cells.

and all injections were administered subcutaneously. The amounts used for each rat were brought up to a total volume of 1 cc. with freshly boiled physiological salt solution and 0.5 cc. was injected twice daily. In all instances a 24 hour period was allowed to intervene before and after the injection of sheep cells, during which time no epinephrine was administered. The method of titration has been described in a previous communication (2).

TABLE II

Hemolysin Formation After Epinephrine-glucose Injections in Suprarenalectomized Rats

Rat No.	Titers			Weight at opera.	Weight at last titer
	5th day	8th day	11th day		
				gm.	gm.
833	1600	160	80	194	178
834	1600	250	100	154	154
835	3000	1000	600	166	166
836	3000	300	160	194	195
837	3000	600	60	230	197
838	4000	700	160	208	183
839	400	400	300	200	181
840	1600	—	—	203	190
841	3000	600	200	185	172
Av. titer....	2355	501	207		

Suprarenalectomized Rats Untreated

843	400	100	60	143	119
844	3000	800	600	200	178
845	1000	400	200	134	120

Normal Rats Untreated

846	3000	200	100	185	191
847	5000	400	200	130	135

The results of these experiments are tabulated in Table I. It is seen that injections of epinephrine diluted with salt solution are followed by a definite rise in antibody formation in suprarenalectomized rats. The average hemolysin titer of the rats in this group was 1/2383 whereas that of the suprarenalectomized rats not treated with epinephrine was 1/858. About 50 per cent of the rats injected with epinephrine yielded titers above 1/2000.

Fig. 1

Fig. 2
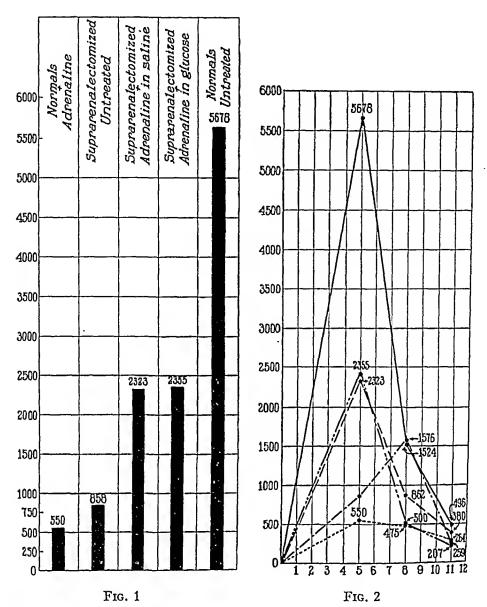
FIG. 1. The average hemolysin titers of normal and suprarenalectomized rats repeatedly injected with epinephrine diluted with physiological salt solution and with isotonic glucose solution.

FIG. 2. The curves of the average hemolysin titers of normal and suprarenalectomized rats repeatedly injected with epinephrine diluted with physiological salt solution and with isotonic glucose solution.

———————— Normal rats.

- - - - - - - - - Normal rats injected with epinephrine.

— — — Suprarenalectomized rats injected with epinephrine diluted with salt solution.

— — – — Suprarenalectomized rats injected with epinephrine diluted with glucose solution.

— — – — Suprarenalectomized rats uninjected.

90

It was observed, however, that the suprarenalectomized rats injected with 0.5 cc. of a 0.9 per cent salt solution also showed a marked rise in antibody formation.

In an effort to eliminate any possible effect of the physiological salt solution used in diluting the epinephrine a second group of experiments was done in which the epinephrine was diluted with isotonic glucose solution (see Table II).   Ten suprarenalectomized rats were injected with 0.2 mg. per kilo of epinephrine (diluted with glucose solution) twice daily during 3 days prior and 4 days subsequent to the injection of the antigen.   Three suprarenalectomized rats received no injection and three normal rats received no injection.   The average titer of the glucose-epinephrine injected rats was 1/2355 or about the same as that of the first group of experiments.   This titer is three times as great as that of the untreated suprarenalectomized rats, but still considerably lower than the average titer in normal rats.

DISCUSSION

It may be concluded from these experiments that the injection of epinephrine raises the hemolysin titer in suprarenalectomized rats and that the effect is not entirely dependent on the salt solution with which the epinephrine is diluted.   The effect of epinephrine diluted with salt solution is not a summation of both epinephrine and salt solution.   We do not believe that the loss in epinephrine resulting from suprarenalectomy is alone responsible for the resulting depression in antibody formation.   There is some evidence however from these experiments that it plays a part in the antibody forming mechanism.

There is a striking difference in the effect of epinephrine injections on the hemolysin formation of normal and of suprarenalectomized rats.   In normal rats, the hemolysin titer was markedly depressed, in suprarenalectomized rats it was raised above the level of untreated suprarenalectomized rats.   An excess of epinephrine supplied to rats possessing their physiological store of epinephrine depresses the mechanism of antibody formation.   Rats, however, deprived of a physiological source of epinephrine and injected with the same amounts as in the previous group are stimulated to increased antibody formation.

CONCLUSIONS

Epinephrine diluted with physiological salt solution or with isotonic glucose solutions and injected in amounts of 0.2 mg. per kilo twice daily into suprarenalectomized rats raises their antibody forming capacity.   This result is not entirely dependent on the effect of the diluting fluid.

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IMMUNOLOGICAL STUDIES IN RELATION TO THE SUPRARENAL GLAND

V. The Effect of Repeated Injections of Solutions Containing Sodium Salts and Glucose on the Hemolysin Formation of Normal and Suprarenalectomized Rats

By J. MARMORSTON-GOTTESMAN, M.D., and DAVID PERLA, M.D.

(From the Division of Laboratories, Montefiore Hospital, New York)

In previous studies (1) on hemolysin formation in normal adult albino rats, it was found that 20 normal rats yielded an average titer of 1/7000, while in 23 additional normal rats the average titer was 1/4323. The average titer of the 43 rats collected in both series was 1/5656, somewhat lower than had previously been estimated with half the number of animals. Seventy per cent of these rats gave titers above 1/2000. An occasional very low reading was noted. From an analysis of the factors responsible for this variation it was observed that the females yielded somewhat higher hemolysin titers than the males (females 1/6850, males 1/4620). Older rats gave a higher antibody titer than young adult rats. Rats of greater weight but of the same age and sex yielded higher titers.

All the rats used in these experiments were bred in the laboratory under the same environmental conditions and were fed on a constant adequate diet. The variations observed in these animals in spite of these constant factors emphasize the necessity of maintaining the factors of age and weight constant and of utilizing large numbers of animals in studies on antibody formation. In our later studies only rats about 3 months of age were used and the results were more uniform.

It was found in previous experiments (2) that suprarenalectomy is followed by a depression in antibody forming capacity most marked during the first week following the operation. Further studies on hemolysin formation in this early period confirmed this observation. Of a series of 23 suprarenalectomized rats injected on the third day

following the operation with 1 cc. of a 10 per cent suspension of sheep cells and tested 5 days later, the average hemolysin titer was 1/858 as compared with 1/5650 of the normal rats. About 70 per cent of suprarenalectomized rats gave titers below 1/2000. Two rats gave high titers corresponding to normal figures. This may be explained by the well known fact that a certain small percentage of suprarenalectomized rats react as normal rats, due to large amounts of accessory cortical tissue (3). Of the suprarenalectomized rats, as of the normal rats, the females gave slightly higher titers than the males.

In the course of experiments on the effect of injections of epinephrine on the hemolysin formation in suprarenalectomized rats (4) it was noted that repeated injections of epinephrine in amounts of 0.2 mg. per kilo raised the hemolysin titer. Small amounts of physiological salt solution raised the titer to about the same level as the epinephrine injected group. This observation suggested the experiments reported in this communication. Studies on hemolysin formation were made in suprarenalectomized rats injected repeatedly with large quantities of a 0.9 per cent salt solution, isotonic sodium acetate, isotonic glucose solution and hypertonic salt solution in order to determine the effect of these substances on antibody production.

Methods

The method of operation, the post-operative care of the animals and the exact procedure of obtaining the blood and titrating the serum has been described in detail in previous communications (5) (2).

In all the experiments reported in this paper, 1 cc. of a 10 per cent suspension of sheep cells was injected intraperitoneally and titers were studied on the 5th, 8th and 11th day following the injection. When suprarenalectomized rats were studied the antigen was injected 48 to 72 hours after operation and the first titer was read on the 7th or 8th day after suprarenalectomy.

The Effect of Injections of Physiological Salt Solution on Hemolysin Formation in Normal and Suprarenalectomized Rats

Four groups of rats were tested. One group of 26 normal rats received two daily injections of 0.5 cc. of physiological salt solution during 3 days prior and 4 days subsequent to the injection of antigen. The average titer in these rats was 1/5516 or about the same as untreated normal rats. A second group of six normal rats

received 5 cc. of physiological salt solution intraperitoneally, once daily during 3 days prior and 4 days subsequent to the injection of sheep cells. The average titer was 1/4133, slightly lower than the titer of normal control rats.*

A third group of thirteen suprarenalectomized rats received 0.5 cc. of physiological salt solution subcutaneously twice daily. The average titer in this group was 1/2630 as compared with 1/858 of the untreated suprarenalectomized rats.

A fourth group of ten suprarenalectomized rats received 5 cc. of physiological salt solution intraperitoneally once daily from the day of operation to the day of the last reading. The average titer in this group was 1/3580, strikingly higher than the titer of untreated suprarenalectomized rats. The general condition of suprarenalectomized rats that had received salt solution injections was excellent. They ate well, exercised vigorously, kept their fur white and sleek and appeared stronger than the uninjected rats. These results indicate a marked beneficial effect from repeated injections of 0.9 per cent salt solution on the general well being and antibody forming capacity of suprarenalectomized rats.

Effect of Injections of Isotonic Sodium Acetate Solution on the Hemolysin Formation of Suprarenalectomized Rats

In an effort to determine the factors responsible for the rise in hemolysin titer in suprarenalectomized rats injected with physiological salt solution, other solutions were used. Isotonic sodium acetate solution was injected into ten suprarenalectomized rats in the same quantities by volume and over the same period of time as those injected with physiological salt solution. The average titer was 1/4260 or about five times as high as the untreated suprarenalectomized rats (1/858). It is apparent therefore that the chlorine ion is not essential

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* Belak and Csereszynes (6) found some stimulating effect of physiological salt solution on the agglutinin formation following the injection of paratyphoid B bacilli in normal rabbits. They injected 0.5 cc. of the solution daily for 14 days after the injection of the antigen. Ringer's solution and isotonic calcium chloride had the same effect. They do not believe the sodium ion was responsible for the rise. Their data were collected on four rabbits. We were unable to demonstrate analogous findings in the case of hemolysin formation in normal rats.

in raising the antibody titer in suprarenalectomized rats. Normal control rats injected with isotonic sodium acetate solution in amounts of 5 cc. per day during 3 days prior and 4 days subsequent to the injection of sheep cells yielded titers slightly lower than untreated normal rats.

Effect of Repeated Injections of Isotonic Glucose Solution on the Hemolysin Titer of Suprarenalectomized Rats

To determine whether the effect of salt solution on the antibody formation in these rats was due to its diuretic effect, 5 cc. of isotonic glucose solution was injected once daily intraperitoneally into eight suprarenalectomized rats during the entire period from the day of operation to the day of the last reading of the hemolysin titer. The average titer of this group was 1/887, or about the same as that of untreated suprarenalectomized rats. In spite of its diuretic action, isotonic glucose did not raise the antibody formation of suprarenalectomized rats. In a group of normal control rats which were injected with isotonic glucose solution there was almost no change in the hemolysin titers from that of uninjected normal rats. The average titer in this group was 1/4500.

The Effect of Concentrated Solution of Sodium Chloride on the Hemolysin Formation of Suprarenalectomized Rats

An effort was made to determine whether sodium chloride alone had the same effect as large volumes of isotonic salt solution. 0.5 cc. of a 10 per cent solution of sodium chloride was injected into eight suprarenalectomized rats once daily from the day of operation during 3 days prior and 4 days subsequent to the injection of sheep cells. The hypertonic solution was extremely irritating. It was observed that the concentrated salt solution had only a slight effect in raising the antibody formation in suprarenalectomized rats. The average titer in this group was 1/1660.

DISCUSSION

It is apparent from these data that injections of isotonic solutions of sodium salts raise the titer of suprarenalectomized rats. This rise in hemolysin titer is not due entirely to the diuretic effect of the large

volume of salt solution injected, since the same volume of glucose solution, an excellent diuretic, has no such effect.   It was found that sodium chloride administered in a small volume of fluid but in the same amount by weight as in the isotonic solutions has only a slight effect in raising the titer in suprarenalectomized rats.   It would seem from these observations that both the fluid and the sodium ion are necessary to bring about a restoration of the antibody forming capacity of suprarenalectomized rats to normal.

Our results are in accord with the findings of Marine and Baumann (7).   They found that the administration of Ringer's solution and isotonic solutions of sodium chloride and sodium acetate increased the duration of life of suprarenalectomized cats about three times.   More concentrated solutions of sodium chloride definitely shorten life.   Isotonic solutions of glucose and glycerol had only a very slight life-prolonging effect.   They concluded from their studies that diuresis is one of the important factors in determining the duration of life.   When the loss of water by diuresis is compensated for by an additional intake, life is prolonged and if not, life is shortened.   There is some indication, they believe, that the loss of sodium is more specific than can be accounted for as a result of a possible acidosis.

It is well known that the sodium ion plays an important rôle in the water exchange in the tissues.   Suprarenalectomy results in a profound disturbance of the water balance in the body.   Following the removal of the glands, there is a loss of fluid through the intestines, a diminution of sodium concentration in the blood (Baumann and Kurland (8)) and an impairment of kidney function.   These factors result in tissue dehydration and a disturbance of the normal cellular physiological activity.   Those tissue elements concerned with antibody production are less able to respond to the stimulation of foreign substances with the production of immune bodies in suprarenalectomized rats than in normal rats.   There may, however, be no actual loss of immune body production but a diminished release of these antibodies into the blood stream.   The restoration of the water balance by the promotion of diuresis with a simultaneous, increased intake of fluid in a form that can be retained in the tissues helps to restore the mechanism of antibody production to the normal.   The rôle of the suprarenal gland in antibody formation appears to be intimately connected with the maintenance of water balance.

The relation of a disturbance in the water balance of the tissues to

TABLE I

Fluid injected	Route of injection	Amount per injection	No. of rats	Titers											
				5th day				8th day				11th day			
				Maximum	Minimum	Mean	Average	Maximum	Minimum	Mean	Average	Maximum	Minimum	Mean	Average
Suprarenalectomized Rats															
none	—	—	21	3000*	0	800	858	4000	60	1200	1576	1000	0	200	259
isotonic sod. chlor.	subcut.	0.5 cc. twice daily	13	12000	160	2000	2630	1600	100	600	709	400	60	200	204
" "	intrap.	5 " once	10	16000	1000	3000	3580	600	40	350	354	200	40	100	110
isotonic glucose	"	5 " " "	8	2000	400	800	887	1000	100	600	600	300	40	100	142
isotonic sod. acet.	"	5 " " "	10	8000	1600	4000	4260	2000	300	1000	990	800	80	200	280
hypertonic sod. chlor.	"	5 " " "	7	2000	1000	2000	1660	1600	600	1200	1240	800	150	300	400
Normal Rats															
none	—	—	43	32000	100	4000	5678	16000	10	1600	1524	2000	10	400	496
isotonic sod. chlor.	subcut.	0.5 cc. twice daily	26	32000	160	4000	5516	12000	80	1600	2000	6000	0	300	582
" "	intrap.	5 " once	6	8000	1800	4000	4133	3000	1000	1000	1500	600	300	300	380
isotonic glucose	"	5 " " "	6	8000	3000	4000	4500	3000	800	1500	1800	800	400	600	580

* A single high reading of 16000 was omitted from average.

antibody formation has hitherto not been noted by previous investigators to the authors' knowledge. This observation is of importance
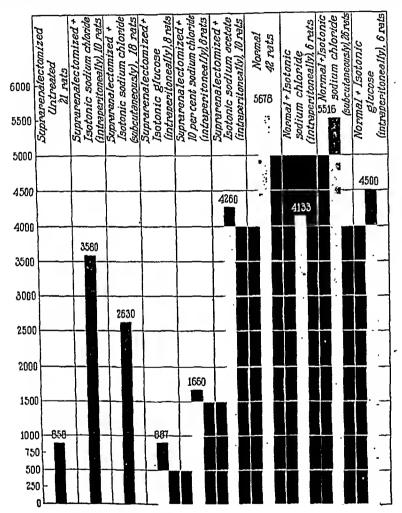
FIG. 1. The effect of solutions containing sodium salt and glucose on the hemolysin formation of normal and suprarenalectomized rats. The columns represent the average hemolysin titer for each group.

in the interpretation of certain phenomena in acute infections. It is a matter of empiricism that "forcing fluids" is beneficial in the treat-

ment of severe infections and in extensive skin burns. It is known
that in many acute infections, and especially in burns, the suprarenal
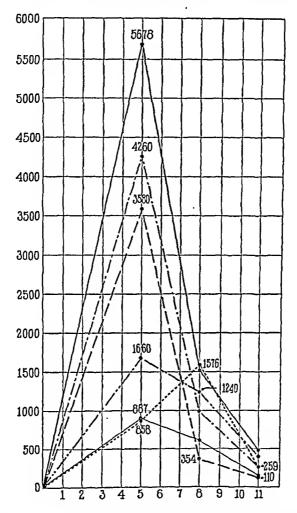
FIG. 2. The curves of the average hemolysin titers of normal and suprarenal-
ectomized rats repeatedly injected with solutions containing sodium salts and
glucose.

———————— Normal rats.
- - - - - - - Suprarenalectomized rats untreated.
— — — Suprarenalectomized rats treated with physiological salt solution.
—— — —— Suprarenalectomized rats treated with isotonic sodium acetate solution.
———————— Suprarenalectomized rats treated with isotonic glucose solution.
— — — — Suprarenalectomized rats treated with 10 per cent sodium chloride
            solution.

glands are severely injured. This injury in turn may result in a disturbance of the tissue water balance and in the antibody forming capacity of the body. This disturbance may be corrected to some extent by parenteral and oral administration of large quantities of physiological salt solution.

In their recent studies on experimentally produced peritonitis in dogs, Orr and Haden (9) injected 1 per cent solutions of sodium chloride and found that the animals lived three times as long as those not given the salt solution. This may be due to the effect of the salt solution on the mechanism of water metabolism disturbed as a result of the suprarenal injury which accompanies a general peritonitis.

SUMMARY

Table I and Figs. 1 and 2 summarize the experiments done on 150 rats. Suprarenalectomy in rats is followed by a striking depression in antibody formation most marked during the first week following the operation. Repeated injections of large amounts of isotonic sodium chloride or sodium acetate solutions restore the antibody titer to normal. The effect of these solutions is not due to diuresis alone, since isotonic glucose solution does not affect the titer, nor to the sodium alone, since hypertonic solutions of sodium chloride in small volume have little influence on the titer. Both the sodium ion and the water volume are necessary in the restoration of the titer.

CONCLUSION

(1) Suprarenalectomy is followed by a disturbance in the antibody forming mechanism of the body. (2) The suprarenal gland plays a rôle in the water metabolism of the tissues. (3) Restoration of the water exchange in the tissues to a normal level is sufficient to raise the antibody forming capacity to normal.

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THE PRODUCTION AND TITRATION OF POTENT HORSE ANTIPNEUMOTOXIN

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In a previous communication[1] it was shown that certain anaerobically produced autolysates of pneumococci contain a poison which produces marked lung lesions when injected intratracheally into small guinea pigs. In a later paper,[2] this poison was shown to be neutralized by the serum of rabbits immunized thereto. These rabbit serums were of relatively low potency, containing approximately 50 neutralizing units per cubic centimeter (0.1 cc. of serum neutralized 5 lethal doses of the autolysate). In the present paper, we wish to report on the production of high titre anti-autolysate serums in the horse[3] and give in detail the method used for their titration.

For the sake of clearness, in this and subsequent papers, the lung-toxic poison will be called "pneumotoxin" and the neutralizing serums for this poison, "antipneumotoxin." We have as yet no absolute proof that the lung-toxic poison is antitoxinogenic, but apparently it is, as it fulfils most of the accepted characteristics of this class of poisons.

EXPERIMENTAL

For the last 8 months, two horses,—Nos. 725 and 726,—have been undergoing treatment with increasing doses of sterile filtrates of the pneumotoxin from Types I, II and III. The first injections of these filtrates were in doses of 0.5 cc. to 1 cc. and produced a marked rise in temperature. For the last 3 months, each of these horses has been

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[1] Parker, J. T., and Pappenheimer, A. M., *J. Exp. Med.*, 1928, 48, 695.

[2] Parker, J. T., *J. Exp. Med.*, 1929, 49, 695.

[3] These horses were immunized to the autolysate filtrates at the Eli Lilly Company in Indianapolis, under the supervision of Mr. W. A. Jamieson.

receiving 400 cc. of the toxic autolysates every 3 or 4 days, without obvious reactions of any kind. Horse 787 has been receiving increasing doses of the pneumotoxin from pneumococcus Type I alone. Horse 382 had been receiving a variety of pneumococcus products, consisting of vaccines, broth cultures of the live pneumococcus, etc., since December 1925, but in November 1928, it was started on the pneumotoxin from Types I, II and III. Horse 685 had been on treatment with pneumococcus vaccine alone since December 1927, but in January 1929 these were discontinued and it was started on inoculations of pneumotoxin from pneumococcus Types I, II and III.

Method of Titration of the Serums for Neutralizing Antibodies to the Lung-toxic Poison

The tests for the presence of neutralizing antibodies were carried out as follows:—Dilutions of the serums in broth were first prepared. 0.1 cc. of the various serum dilutions, or broth instead of the serum dilutions, were pipetted into separate precipitin tubes which were kept in ice water. To each tube 0.9 cc. of chilled pneumotoxin was added and the contents immediately mixed. Heavy vaseline seals were then added to all the tubes, care being taken to avoid the formation of bubbles between the surface of the mixture and the vaseline. The tubes were then left at room temperature in the dark for 2 hours. After this, they were again placed in ice water, the vaseline seals removed and the contents of each tube pipetted into a chilled Wassermann tube. One or two guinea pigs were then injected intratracheally with 0.2 cc. of each preparation. To avoid the danger of oxidation by exposure to air, the vaseline seals were not removed until immediately before the inoculation of the guinea pigs. Guinea pigs weighing from 190 to 210 gm. were used in all the experiments.

Pneumotoxin from Types I, II or III were employed, autolysates from one type only being used in each experiment. These toxins were of such strength, that 0.2 cc. of mixtures of 0.9 cc. of toxin and 0.1 cc. of broth (0.18 cc. of toxin) invariably killed guinea pigs of 200 to 210 gm. in less than 24 hours with typical symptoms and autopsy findings.

One unit of toxin is the amount which, when injected intratracheally, will kill a guinea pig weighing 200 to 210 gm. in from 4 to 24 hours,

with typical symptoms and autopsy findings; while one unit of anti-toxin represents the smallest amount of serum which is necessary to protect a guinea pig of the same weight against one unit of toxin when the mixture is injected intratracheally.

Results from These Neutralization Experiments

The strength of the horse serums in neutralizing antibodies for the lung-toxic poison appears to depend quite regularly on the amount of pneumococcus toxic autolysate the horses have received. The serums of Horses 725 and 726, the horses most highly immunized to the toxic autolysates, have shown a progressive and rapid increase in their content of neutralizing antibodies during the last 3 months (an increase of from 5000 to 40,000 units).

On the other hand, serums from several bleedings from Horse 787, which had recently been started on pneumococcus autolysate injections, and from Horses 382 and 685, which had been treated with a variety of pneumococcus products for several years and only recently with injections of toxic autolysates, all showed relatively small amounts of neutralizing antibodies. However, recent tests carried out with a concentrated preparation from the latest bleedings from Horses 382 and 685, show an increase in these antibodies. (See Table I.)

Effect of Normal Horse Serum, Antipneumococcus Horse Serum or Antipneumococcus Horse Serum Concentrated by the Felton Method[4] on the Lung-Toxic Autolysates

Neither normal horse serum nor unconcentrated antipneumococcus horse serum containing 500 protective units per cubic centimeter to Pneumococcus I had any detoxifying effect on the toxin when used in 1–10 dilution—0.1 cc. of serum added to 0.9 cc. of toxin—and set up under the same conditions as the other mixtures. The antipneumococcus serum concentrated by the Felton method and containing 1000 protective units per cubic centimeter to Pneumococcus I, detoxified the toxin when used in 1–20 dilution, but had no effect on the toxin when used in 1–50 dilution.

Experiment I.   Table I

Serum Tested:

   787.   4th bleeding.
   725.   7th bleeding.
   31222.   Concentrated preparation from 6th bleeding of horses 725 and 726.
   31221.   Concentrated preparation from the 86th bleeding of Horse 382 and
            the 13th bleeding of Horse 685.

The in Vitro Neutralization of Lung-Toxic Autolysates with Autolysate Horse Serums

Pig No.	Weight	Serum tested	Final dilution of serum	Symptoms	Died or survived	Amt. consolidation in lungs	Calculated units of antitoxin per cc.
350	192	787	1–50	0	S.		
312	194	787	1–100	++	D <18 hrs.	+++	250
327	208	787	1–200	+++	D <18 hrs.	+++	
317	192	725	1–1000	0	S.		
308	206	725	1–2000	0	S.		20,000
346	190	725	1–4000	0	S.		
315	206	725	1–8000	+++	D <18 hrs.	++	
322	190	31222	1–2000	0	S.		
311	192	31222	1–4000	0	S.		20,000
314	204	31222	1–8000	++	D <48 hrs.	++	
321	206	31222	1–8000	+++	D <18 hrs.	+++	
325	190	31221	1–200	0	S.		
323	202	31221	1–400	0	S.		2,000
349	202	31221	1–1000	+++	D <40 hrs.	+++	
324	204	31221	1–2000	++	S.		
307	210	—	—	+++	D <18 hrs.	++	
309	208	—	—	+++	D <18 hrs.	++	
358	206	—	—	+++	D <18 hrs.	+++	

This table is self explanatory.

Table I shows the results of a typical experiment made to determine the strength of these neutralizing substances in the serum of immunized horses.   There is one discrepancy in this experiment, in that Guinea pig 349, which received twice as much of Serum 31221 in its toxin-serum mixture as did Guinea pig 324, died; while the latter pig, although extremely sick for several days, survived.   In tests carried out with the greater serum dilutions, discrepancies of this sort occa-

sionally occur. We believe that these discrepancies are due to the greater susceptibility to the toxin of a small percentage of pigs (approximately 10%), in which instances the lethal dose of toxin may be $\frac{1}{2}$ to $\frac{1}{4}$ the usual lethal dose. Therefore, for accurate results in the titration of a serum, it is necessary to use several pigs for each of the final dilutions of serum.

In a previous paper, it was shown that the pneumotoxin for Types I and II were antigenically similar. This experiment, Table I, brings out a point which we have recently confirmed several times, *viz.*, the pneumotoxin from Pn. Type III is also antigenically similar to toxins from Types I or II. (See Pig 350 in the table.)

Toxin used was a toxic autolysate from Pn. Type III. 0.9 cc. of the toxin was added to 0.1 cc. of the diluted serum, or to 0.1 cc. of broth. After standing for 2 hours at room temperature under vaseline seal, guinea pigs were injected intratracheally with 0.2 cc. of the mixtures.

CONCLUSIONS

1. The serum of horses immunized with increasing doses of certain anaerobically produced autolysates of pneumococci contain potent neutralizing antibodies for the pneumotoxin.

2. The method for the *in vitro* titration of these horse antipneumo-toxic serums is given.

BIOMETRY OF CALCIUM, INORGANIC PHOSPHORUS, CHOLESTEROL, AND LECITHIN IN THE BLOOD OF RABBITS

IV. Effects of a Malignant Tumor

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The present paper is part of a study of the relations of certain blood constituents in animals as affected by various types of environment and disease (1, 2, 3). The experiments to be reported deal with the variations of calcium, inorganic phosphorus, lecithin and cholesterol in the blood of animals inoculated with a malignant neoplasm (4).

Material and Methods

The animals used were male rabbits from 6 to 8 months old at the beginning of the experiment. While under observation, the animals were caged separately, and kept in a well lighted (sunlight), well ventilated room with a southern exposure. The rooms were heated during the colder weather. The diet of the animals consisted of hay, oats, and cabbage.

Three groups of 10 animals each were inoculated in one testicle with 0.3 cc. of a tumor emulsion. Examinations of the blood were made at weekly intervals for a period of 2 months. During this time, some animals died; those that survived were then killed and autopsied.

The results for the control animals will be omitted as the general trend and variation of results are comparable to the group previously reported (2). Moreover, a comparison of the values obtained for animals that recovered with those for animals that died is more significant than a comparison made with normal values.

At the conclusion of the experiment, the 30 animals comprising this series were divided into 2 groups: (1) those animals in which death was due to the neoplasm, and (2) those recovering from the tumor inoculation. The results obtained from these 2 groups of animals are presented in the present paper. The results for the

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12 animals in which death was due to the tumor (Group I) are presented in Table I, while the results of the 18 animals recovering from the tumor inoculation (Group II) are presented in Table II. Each table is divided into 7 dates of record, the first recorded examination, −14 days, are the results obtained 14 days preceding inoculation; second, the results obtained on the day of inoculation; third, the results +14 days after inoculation. The subsequent 4 determinations were made at 7 day intervals. The recorded values at "time of death" does not mean all animals died 42 days after inoculation, but summarizes the results obtained from these animals a day or two preceding expiration. The data presented in this paper are derived from experiments, the procedures of which were described in detail in a preceding paper (1). In all text-figures and tables the lipoid phosphorus is calculated and presented as lecithin.

The pertinent features of the experiment may be summarized as follows:

Group	No. of animals	No. of examinations	Date of inoculation	Observation period
A	10	99	Nov. 17, 1927	Oct. 27, 1927 to Jan. 12, 1928
B	10	99	Jan. 19, 1928	Jan. 5, 1928 to Mar. 22, 1928 ·
C	10	76	Mar. 27, 1928	Mar. 15, 1928 to May 22, 1928

Results

The results of the observations made in this experiment are presented in the form of tabulated summaries, Tables I to III, which are supplemented by a series of graphs, Text-figs. 1 to 10. The mean values contained in Tables I and II have been smoothed by the formula $\dfrac{A + 2B + C}{4}$ and are presented in the graphs in terms of per cent variations from standard mean values, using for this purpose values obtained for animals living in the open laboratory throughout the year. These results were reported in a previous paper (2) and shall serve also as a basis of comparison for certain ratio values obtained in the present experiment.
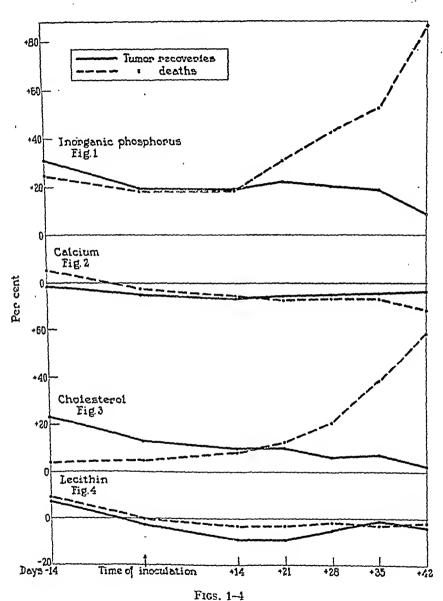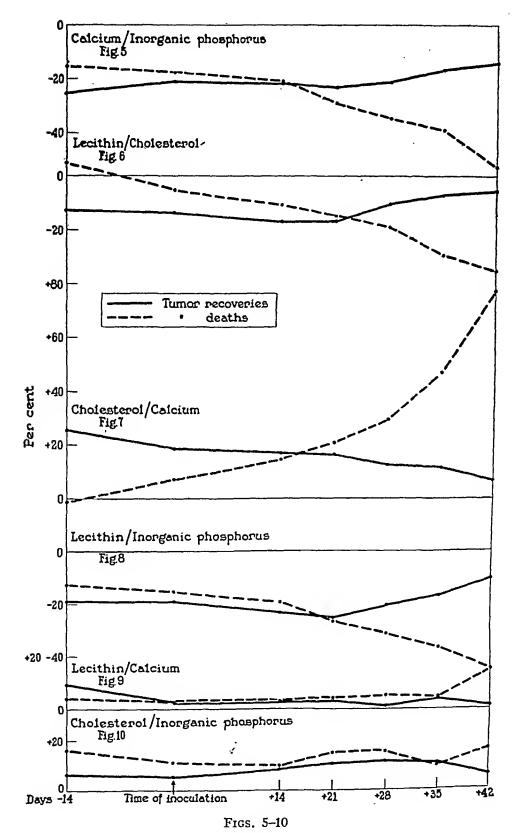
TABLE I

Group I. Tumor Deaths

Days	Inorganic phosphorus			Calcium			Cholesterol			Lecithin		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
	ms. per 100 cc.	*ms.*	*per cent*	*ms. per 100 cc.*	*ms.*	*per cent*	*ms. per 100 cc.*	*ms.*	*per cent*	*ms. per 100 cc.*	*ms.*	*per cent*
-14	5.59 ± 0.22	1.11	19.87	16.5 ±0.29	1.47	9.03	60.6 ±1.67	8.59	14.17	129 ±5.08	26.1	20.33
Inoculated	5.52 ± 0.25	1.29	23.37	15.0 ±0.17	0.87	5.80	60.0 ±1.61	8.26	13.77	113 ±4.30	22.1	19.56
+14	5.37 ± 0.12	0.63	11.75	14.8 ±0.18	0.95	6.42	63.7 ±1.27	6.51	10.22	118 ±5.47	28.1	23.81
+21	5.96 ± 0.22	1.11	18.62	14.7 ±0.13	0.67	4.56	64.4 ±1.75	8.97	13.93	108 ±5.82	29.9	27.68
+28	7.19 ± 0.91	4.65	64.67	14.4 ±0.23	1.17	8.13	69.0 ±2.09	10.72	15.54	123 ±3.29	16.9	13.74
+35	6.34 ± 0.38	1.95	30.76	15.4 ±0.37	1.88	12.21	80.5 ±6.09	31.28	38.86	110 ±1.42	7.3	6.64
Time of death	8.67 ± 1.10	5.67	65.39	13.9 ±0.26	1.33	9.57	91.5 ±6.42	32.98	36.04	120 ±3.82	19.6	16.33

TABLE II

Group II. Tumor Recoveries

Days	Inorganic phosphorus			Calcium			Cholesterol			Lecithin		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
	mg. per 100 cc.	mg.	per cent	mg. per 100 cc.	mg.	per cent	mg. per 100 cc.	mg.	per cent	mg. per 100 cc.	mg.	per cent
−14	6.13 ±0.11	0.67	10.92	15.4 ±0.29	1.85	12.01	71.6 ±1.90	11.93	16.62	127 ±4.27	26.87	21.16
Inoculated	5.29 ±0.10	0.65	12.29	15.0 ±0.15	0.96	6.40	64.1 ±1.45	9.13	14.24	114 ±4.79	30.16	26.46
+14	5.67 ±0.15	0.93	16.40	14.5 ±0.14	0.91	6.28	63.1 ±0.76	4.80	7.61	107 ±4.38	27.57	25.77
+21	5.75 ±0.09	0.54	9.39	15.0 ±0.18	1.07	7.13	65.3 ±1.29	7.86	12.04	105 ±3.11	19.02	18.11
+28	5.81 ±0.08	0.50	8.61	14.7 ±0.11	0.68	4.63	62.0 ±1.39	8.48	13.68	115 ±3.49	21.36	18.57
+35	5.68 ±0.16	0.95	16.73	15.2 ±0.26	1.57	10.33	63.8 ±1.04	6.33	9.92	120 ±2.89	17.59	14.66
+42	5.05 ±0.14	0.83	16.44	15.1 ±0.17	1.03	6.82	59.7 ±1.34	8.21	14.18	114 ±2.52	15.40	13.51

TABLE III

Coefficients of Correlation

	rP. Ca.	rP. Chol.	rP. Lec.	rCa. Chol.	rCa. Lec.	rChol. Lec.	
Deaths from Tumor. Group I							
Trend............	−0.654 ±0.12	+0.867 ±0.05	+0.165 ±0.21	−0.511 ±0.16	+0.290 ±0.20	−0.083 ±0.22	N = 7
Individual mean...	−0.197 ±0.18	+0.653 ±0.12	−0.571 ±0.13	−0.200 ±0.18	+0.768 ±0.07	−0.066 ±0.20	N = 12
Series............	−0.477 ±0.06	+0.537 ±0.05	−0.138 ±0.08	−0.319 ±0.07	+0.277 ±0.08	−0.072 ±0.09	N = 83
Recoveries from Tumor. Group II							
Trend............	+0.113 ±0.21	+0.329 ±0.19	+0.337 ±0.19	+0.542 ±0.16	+0.729 ±0.11	+0.546 ±0.16	N = 7
Individual mean...	−0.400 ±0.13	−0.428 ±0.13	−0.350 ±0.14	+0.171 ±0.15	+0.618 ±0.10	+0.066 ±0.22	N = 18
Series............	−0.188 ±0.06	+0.206 ±0.05	−0.192 ±0.06	+0.086 ±0.07	+0.305 ±0.05	+0.029 ±0.07	N = 122

FIGS. 1–4

FIGS. 5–10

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DISCUSSION AND CONCLUSIONS

An examination of the results given in Tables I and II and the curves in Text-figs. 1 to 4 show certain striking differences between animals which died from the tumor and those that recovered. The differences were exhibited chiefly in the variation and trend of inorganic phosphorus, calcium, and cholesterol; the lecithin remaining more or less constant in both groups of animals.

Inorganic Phosphorus.—At the beginning of the experiment, the inorganic phosphorus (Text-fig. 1) in Group I was slightly lower than that obtained for Group II. At the time of inoculation both groups showed a decrease in inorganic phosphorus but the relative position was the same. The next examination, 14 days after inoculation, gave values of inorganic phosphorus which coincided. From this period, Group I gave increasing values for inorganic phosphorus and at the end of the experiment the results were 60 per cent higher than those recorded at the time of the first examination. Group II showed a steady decrease in inorganic phosphorus, the final level for this substance being 20 per cent lower than that obtained at the first examination. The inorganic phosphorus exhibited the highest per cent variation.

Calcium.—The calcium (Text-fig. 2) in Group I, at the −14 day period, was slightly higher than that found in Group II. With a gradual decrease in trend, this position was maintained until after the +14 day period at which time the position of the 2 groups was reversed. From this time, the results for Group I showed a more or less constant level until at the time of the last examination the values obtained for calcium were decidedly lower than those obtained for Group II. The calcium in the blood serum of Group II exhibited an uninterrupted increase beginning 14 days after inoculation and continuing throughout the remaining period of the experiment.

Cholesterol.—The cholesterol (Text-fig. 3) content of the whole blood in Group I was practically 20 per cent higher than that found in Group I at the −14 day examination. From this high level, the cholesterol in Group II showed a marked decrease which continued throughout the remainder of the experiment. At the

time of inoculation, the cholesterol content of the whole blood in Group I was practically the same as that of the preceding examination. From this time, however, the cholesterol increased rapidly, the last value being 55 per cent higher than that obtained for Group II.

Lecithin.—The lecithin (Text-fig. 4) level for Group I was slightly higher than that found in Group II throughout the entire experiment, with the exception of the results obtained at the +35 day period. At this time, the values for Group I were slightly lower than those for Group II. From the beginning of the experiment, both groups exhibited a gradual decrease in lecithin which continued until 14 days after inoculation. During the next 7 days both groups remained practically constant. From this period, Group II showed a gradual increase in lecithin, reaching a maximum on the +35 day examination. At the time of the last examination, however, the values were again lower than Group I. The lecithin for Group I throughout this same period maintained an almost constant level.

Ratios

The ratio curves (Text-figs. 5 to 10 inclusive) are presented in terms of per cent variation of the smoothed values from standard ratios (2).

The calcium-inorganic phosphorus ratio (Text-fig. 5) of Group I at the —14 day period was almost 10 per cent higher than that found in Group II. At the time of inoculation, the ratio for Group I showed a slight decrease while in Group II the ratio was markedly increased. From the time of inoculation throughout the remainder of the experiment, the ratio of calcium to inorganic phosphorus for Group I continued to decrease, the last determination being 36 per cent lower than that found in Group II. This low value was due to both an increase in inorganic phosphorus and a decrease in calcium. The trend of the ratio for Group II following inoculation showed a decrease of about 2 per cent at the +21 day period. From this time on, however, the ratio in this group continued to rise, reaching its highest value at the time of the last examination.

The lecithin-cholesterol ratio (Text-fig. 6) for Group I began with

a value about 4 per cent above the standard. From this time throughout the entire experiment, the ratio for Group I showed an uninterrupted decrease and at the last examination was 29 per cent below Group II, the decrease being affected chiefly by the marked increase in cholesterol occurring in this group of animals. At the −14 day examination, the lecithin to cholesterol ratio in Group II was some 16 per cent lower than that found in Group I. Throughout the next 28 day period, the ratio trend showed a slight decrease. During the next 7 days there was a tendency toward stabilization. Following this period of stabilization, however, the ratio trend in Group II showed a marked increase which continued throughout the remaining period of the experiment.

The cholesterol-calcium ratio (Text-fig. 7) for animals in Group I began with a value slightly under the standard. There occurred a gradual increase in the ratio up to the +14 day period. Following this, the ratio increased very rapidly and at the time of expiration the value for the cholesterol-calcium was 80 per cent higher than that found at the −14 day examination. This high ratio value was due to both an increase in cholesterol and a decrease in calcium. The cholesterol-calcium ratio for animals in Group II at the −14 day examination was 25 per cent higher than that found in the animals of Group I. From this initial value, the ratio trend continued to decrease gradually until the +21 day period. Following this date, the decrease which continued throughout the remainder of the experiment, was more rapid and at the final examination was practically 18 per cent lower than the first determination. A more detailed discussion of calcium-cholesterol relationship will be presented in a subsequent paper.

The trend of the lecithin-inorganic phosphorus ratio (Text-fig. 8) for both groups was somewhat similar to that of calcium-inorganic phosphorus. At the −14 day examination, Group I showed a ratio level about 5 per cent higher than Group II. With a slight decrease in trend, the 2 groups maintained practically the same relationship until the +21 day period when their ratio positions were reversed. From this date, the ratio for animals of Group I showed a rapid decrease while that for Group II gave increasing

values. This change in position for both groups of animals can be attributed chiefly to the variation of inorganic phosphorus.

The lecithin-calcium ratio (Text-fig. 9) for animals in Group I was slightly lower than that found in Group II. At the time of inoculation and throughout the following +14 day period, the ratios for both groups coincided. At the time of the +21 day examination, the ratio for Group I was slightly higher than that found in Group II. This position continued throughout the remainder of the experiment and at the time of the last examination the animals of Group I gave a ratio for lecithin-calcium 14 per cent higher than that of Group II.

The cholesterol-inorganic phosphorus ratio (Text-fig. 10) showed the least per cent variation. Group I which at the —14 day examination gave a ratio value 10 per cent higher than Group II, showed a gradual decrease in trend until the +14 day period. During the next 14 days a slight increase occurred to be followed by a rapid decrease. This decrease occurred at the +35 day examination and was the only time during the experiment when the cholesterol to inorganic phosphorus ratio for Group I was below that of Group II. This final examination resulted in a .value for Group I which was 10 per cent higher than that found in Group II. During the 14 days preceding inoculation, the trend of the cholesterol-inorganic phosphorus ratio for Group II paralleled that of Group I. Following inoculation the ratio for Group II gradually increased, reaching its highest level on the +28 day examination. From this time, the ratio decreased, the decrease being more rapid throughout the last 7 days of the experiment.

It will be noted that in all ratio curves the change in position or trend occurred about 21 days after inoculation. This corresponds in a measure with the clinical course of the disease, for it is about this time that the ultimate fate of the animal is determined; from this point onward the tumor either pursues a malignant course or is gradually brought under control and recovery begins.

In the following table a comparison is made between the two groups of animals with respect to their ratio positions at the end of the experiment, 1 representing the highest and 4 the lowest value.

Ratio Positions of Tumor Animals

Group	Ca./P.	Lec./Chol.	Chol./Ca.	Lec./P.	Chol./P.	Lec./Ca.
II	1	1	4	1	4	1
I	4	4	1	4	- 1	1

When compared with a previous report on the influence of light environment (3), it will be noted that with the exception of the cholesterol to inorganic phosphorus ratio, the animals in Group I occupy a position similar to that of animals exposed to the ultra-violet light, while Group II occupies a position similar to animals living in total darkness.

Correlation

The coefficients of correlation for both groups of animals calculated on the basis of trend, individual mean values, and for each group series, are presented in Table III. It will be noted that the highest negative r inorganic phosphorus-calcium occurred in the animals of Group I. In Group II the r calcium-cholesterol were all positive while in Group I these same coefficients were all negative and of a somewhat greater magnitude. The r cholesterol-lecithin were all positive in Group II. These values while rather small were all negative in Group I.

Summarizing the results of this experiment, it will be noted that animals in which the tumor proved most malignant (Group I) there occurred a marked increase in inorganic phosphorus of the blood serum and cholesterol of the whole blood and a decrease in serum calcium. The ratios of cholesterol to calcium and lecithin to calcium increased in trend following inoculation, while the ratios of calcium to inorganic phosphorus, lecithin to cholesterol, and lecithin to inorganic phosphorus showed a marked decrease in trend. The animals in Group II exhibited trends and ratios of these 4 blood constituents similar to the control animals (2).

SUMMARY

Experiments are reported in which it was shown that the calcium, inorganic phosphorus, cholesterol, and lecithin in the blood of

normal rabbits were influenced by inoculation with a malignant tumor (4).

Animals in which death was due to tumor gave results on which the following conclusions are based:

1. A marked increase in inorganic phosphorus of blood serum and cholesterol of whole blood followed inoculation.

2. The ratios of cholesterol to calcium and lecithin to calcium increased in trend, while the calcium to inorganic phosphorus, lecithin to cholesterol, and lecithin to inorganic phosphorus ratios showed a marked decrease in trend.

3. The r cholesterol-lecithin were all negative.

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BLOOD AND BONE MARROW CELLS OF THE DOMESTIC FOWL

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PLATE 6

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The development of the supravital technique for the study of living cells has provided a new and interesting method for the cytological study of various animal tissues. This paper concerns the application of such methods to the blood and bone marrow cells of the domestic fowl. White Leghorn, Plymouth Rock and Black Jersey Giant roosters have been used in these experiments.

Direct Method of Counting

The problem of securing an accurate count of the white blood cells of the fowl has presented three difficulties. The avian red blood cells are nucleated, hence any method using the usual diluting fluids containing acetic acid will not permit discrimination between the red blood cell nuclei and the white blood cells. The second obstacle to an accurate count is the character of the thrombocytes. These structural elements in the fowl are about the size of lymphocytes and some of them may be confused either with red blood cells in which there is little hemoglobin, or with lymphocytes. The third factor is the rapidity with which coagulation occurs in the blood of the fowl.

Blain (1) has recently introduced a direct method for making total white blood cell counts on avian blood. He used two diluting fluids; Solution 1 contained neutral red 1:5000 made up in Locke's solution and adjusted to a pH of 7.4. The blood was first diluted with this solution at a temperature of 39°C., until the red cell pipette was one-half full. The mixture was shaken for 15 seconds, at the end of which time the pipette was filled with Solution 2, consisting of 12 per cent formalin in Locke's solution at pH 7.4. Shaking was then resumed for from 2 to 3 minutes. In this way he stated that every white cell had taken up sufficient neutral red to make possible its identification from the red blood cells. The latter· took up no neutral red.

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Prior to Blain's work the only method of estimating the number of white blood cells of avian blood was by indirect calculation, in which the total number of red and white corpuscles was determined in the counting chamber.   Then, in films, the ratio between the number of red and white blood corpuscles was determined.   From the total number of blood cells and from this ratio the number of white blood corpuscles per cubic millimeter may be calculated.   This indirect method is subject to great error chiefly for two reasons.   It presupposes an absolutely even distribution of red and white blood cells, which actually rarely obtains.   The second source of error is in the thrombocytes.   The size and character of these elements make their separation from lymphocytes in the counting chamber exceedingly difficult or impossible.

Blain (2) stated in a further communication that he identified no structures in avian blood corresponding to the platelets of mammals. It would appear, then, that the structures which other investigators, among them Ellermann (3), Albertoni and Mazzoni (4), Sugiyama (5), and which we ourselves consider to be thrombocytes have been included either in the red blood cell counts or in the white blood cell counts of Blain.

The method employed for the counting of the white blood cells in the studies reported in this paper has also made use of the vital staining properties of neutral red.   A single diluting fluid has been devised, consisting of 25 mgm. of neutral red in 100 cc. of 0.9 per cent sodium chloride solution.   This solution is filtered once and is kept at room temperature.   It is preferable, but not essential to have the fluid warmed to body temperature at the time of use.   The ordinary red blood cell pipette is used.   The blood from a puncture of the wing vein or of the comb is drawn to the 0.5 mark and immediately diluted to the 101 mark, giving a dilution of 1:200.   The pipette is shaken for 4 or 5 minutes as in ordinary blood cell counts.   The counting chamber is filled in the usual manner.   We have found it quite unnecessary to have the fluid at a definite pH or to introduce other complicated procedures.

It has been found that with this diluting fluid the polymorphonuclear elements and the monocytes stain in a characteristic manner, so that they can be recognized in the counting chamber with an 8 mm. lens and No. 10 objectives.   The lymphocytes and thrombocytes are

not appreciably stained with the dye. The polymorphonuclear cells are characteristic in that the cytoplasmic border stains heavily and gives the appearance of a very dark, heavy rim to these cells. The monocytes take the dye more diffusely and do not have the deeply stained border. The lymphocytes and thrombocytes can be seen in the chamber but remain almost entirely unstained. Those red blood cells which have a small amount of, or no hemoglobin likewise fail to stain. The last three elements, lymphocytes, thrombocytes, and red blood cells without appreciable hemoglobin cannot be distinguished with any degree of accuracy in the counting chamber. The white blood cell count is made in the following manner: The total number of polymorphonuclear cells and monocytes is counted in the whole area of the ruled portion of the chamber (0.9 cu. mm.). This value taken together with the percentage of these combined elements obtained from the differential count permits a calculation of the total leucocyte count, exclusive of thrombocytes. An example will clarify this explanation.

Total number of polymorphonuclear cells and monocytes in 0.9 cu. mm.. 7200 cells
Total number of polymorphonuclear cells and monocytes in 1 cu. mm.. 8000 cells
Differential leucocyte count
   Polymorphonuclear eosinophiles......................... 50 per cent
          "   neutrophiles......................... 4
          "   basophiles........................... 2
   Monocytes.. 10
    Total polymorphonuclear cells and monocytes........... 66 per cent
   Small lymphocytes.................................... 30
   Large   "   4
                                      100 per cent

Therefore,   66% of total count = 8000 cells

$$100\% \text{ " " " } = \frac{8000}{66} \times 100 = 12{,}121+ \text{ white blood cells per cu. mm.}$$

By this method the difficulties of confusing the thrombocytes with the white blood cell count or with the erythrocyte count are overcome. There is also the advantage that the red blood cells may be counted in the same chamber as the leucocytes. This is practicable in the chicken because of the relatively low erythrocyte and relatively high leucocyte counts.

It should be stated here that the above method has been employed in this laboratory on many animals over a period of two years with con-

sistent results. The accuracy has also been tested by using the method on human blood, where entirely consistent results were obtained. The method was tried on a case of human lymphatic leukemia with a count of 60,000 cells. None of the lymphocytes contained any significant amount of dye, whereas the polymorphonuclear leucocytes and monocytes stained as they do in chickens' blood. It is pointed out that the method is readily adaptable for the counting of human blood, in which there are many normoblasts. I recall a case of sickle cell anemia in which there were 40,000 to 70,000 normoblasts per cubic millimeter of blood. The usual acetic acid diluting fluid does not distinguish these cells from leucocytes and thus a false leucocyte count was obtained. Likewise the normoblastic showers occurring in pernicious anemia are apt to lead to an erroneous white blood cell count unless a method of the type here outlined is resorted to.

Supravital Studies

Because of the diversity of opinion concerning the character of the cells in the blood of the domestic fowl, it seems necessary to give in some detail descriptions and illustrations of the types of cells encountered. It should be pointed out at this time that the following descriptions of cells apply only to the domestic fowl. Other kinds of birds, as pigeons, doves, etc., show a somewhat different picture.

Detailed observations on the blood and bone marrow cells of the chicken have been made by the use of the supravital technique and are reported in this communication. This is now a generally accepted method of great value to the cytologist for the study of living cells. It yields information concerning the motility, phagocytic power, mitochondrial content, fragility, and life history of the cells, which cannot be obtained by studying fixed smears or sections. The method has been used for many years by isolated investigators and has been more recently advocated and described by Simpson (6), Sabin (7), and others. For detailed descriptions of the technique, their papers should be consulted. In these studies neutral red and Janus green have been used as in the method given by Sabin (7).

Descriptions of Blood Cells

Red Blood Corpuscles (Fig. 11).—These cells in the chicken are all nucleated. They are oval in shape and considerably larger than the

red blood cells of man. The nucleus is usually slightly irregular and roughly oval in shape, and is smaller than that of a small lymphocyte. It is about the same size, or somewhat smaller than that of a thrombocyte. The nuclei of the normal red corpuscles are distinct from those of any other cells in that most of them are clear and show very little nuclear structure. Occasionally one finds a nucleus of a red blood corpuscle which is somewhat shrunken, more round than usual, and which has a more distinct, reticular structure. Around the nuclei of red blood corpuscles one almost invariably finds a few bluish-green, rod-shaped mitochondria which stain specifically with Janus green. The cytoplasm also contains from none to several small, reddish-brown bodies which are usually near the nucleus, but they often move about and may be far out near the cell border. In the supravital film, cells are sometimes found which obviously belong to the erythrocyte series, but in which there is no hemoglobin or a barely perceptible amount (Fig. 8). These cells have been confused with thrombocytes (Figs. 7, 9). However, careful scrutiny will always reveal an intact cellular membrane. The cytoplasm is much more abundant than that of the thrombocyte and the nucleus is distinct. These we have called degenerating forms of red blood cells. Clinging to their nuclei one can often see refractive vacuoles which are rarely stained and which are different from the stained vacuoles one sees in thrombocytes.

'Thrombocytes' (Figs. 7, 9).—These cells in the fowl are nucleated and are about the size of small or intermediate lymphocytes. They are distinctly smaller than erythrocytes. Their configuration is usually irregular in contrast to the round lymphoid cells and the oval red blood cells. The nuclei are distinguished by a very pronounced splotching which is so marked that it often appears vaculated. This is in contrast to the clear, almost structureless nuclei of the erythrocytes, and to the softer, more cloudy appearance of the nuclei of the lymphocytes. The cytoplasm of thrombocytes is often quite clear at first (Fig. 9), but after a few minutes' exposure to neutral red and Janus green, vacuoles (one or a few) develop at one or both ends of the nuclei (Fig. 7). These vacuoles stain a muddy brown color, are non-refractive, and increase in size with longer exposure to the dye. Sometimes a few very delicate, barely perceptible mitochondria appear in the perinuclear area. These bodies are much less constantly found and are not as large or as conspicuous as in lymphocytes.

Thrombocytes occur characteristically in clumps of two, three, or many cells, but are also found singly. In this respect they behave as do the blood platelets of mammals.

Polymorphonuclear Eosinophiles (Fig. 1).—These cells are numerous in the blood stream and, as has been shown by Moore (8) and others, are the elements which are specifically increased in fowl typhoid. They have a lobulated nucleus with a reticular chromatin network. The cytoplasm is filled with rod-shaped, specific granules which stain red with eosin in fixed preparations and stain a golden-yellow with neutral red in supravital films. These rods are not very uniform in size and shape. Some of them are short and almost round; others are sometimes club-shaped. The majority of them, however, are uniform in their size and staining reactions. After a few minutes' exposure to the dye, one or more vacuoles may appear in the cytoplasm. These vacuoles stain a reddish-orange color and increase in size with longer exposure to the dye. Mitochondria are rarely seen. When present, they are delicate filaments or dots staining specifically with Janus green. The polymorphonuclear eosinophiles are actively motile cells. They are probably comparable, in their function, to the polymorphonuclear neutrophiles of human blood.

Polymorphonuclear Cells with Pseudo-eosinophilic Granules (Fig. 2).—These elements have been commonly referred to in the literature as true eosinophiles, as eosinophiles with round granules, and as neutrophiles. They differ from the eosinophilic cells with rods not only in the shape of the granules, but also in their chemical reaction to the dye. The granules are really pseudo-eosinophilic. They take very little of the color in neutral red preparations. The granules are uniform in size and color and are quite small. They rarely fill the cytoplasmic area. The granules are very similar to the granules of amphophiles or neutrophiles of rabbits. The nucleus is usually bilobed. Mitochondria are commonly present among the specific granules. The cells are rarely as actively motile as the cells with eosinophilic rods.

Basophiles (Fig. 4).—The cells with specific basophilic granules are about the same size as those with eosinophilic rods. The nucleus is usually a single, irregular or round mass, but may be lobulated. The nucleus shows a diffuse chromatin distribution. The cytoplasm is filled with red specific granules. The cells are only slightly motile.

Monocytes (Fig. 5).—These are conspicuous cells in the chicken's blood. They vary somewhat in size from that of the polymorphonuclear cells to slightly larger. The nucleus is commonly indented or horseshoe-shaped but may be round or lobulated. Mitochondria are abundant, small, and may be in the shape of filaments, rods, or dots. The cytoplasm possesses a hazy appearance in contrast to the clear, glassy cytoplasm of lymphocytes. Neutral red bodies are prominent features and are often grouped together, forming a rosette in the bay of the nucleus. These neutral red bodies (segregation apparatus) increase in size with longer exposure to the dye. They stain a brick-red color and are almost all non-refractive. These cells are motile and have the same type of motility as has been described by Sabin (7) for the monocytes of human blood.

Lymphocytes (Figs. 6, 10).—Small, intermediate, and large lymphocytes are easily distinguished in fowl blood. The small lymphocytes are much more numerous than the larger forms. They are about the size of human small lymphocytes, but appear much smaller because of the larger red blood cells with which to compare them. They are similar in all respects to the lymphocytes of human blood. The nucleus is round or slightly indented and is relatively large in proportion to the amount of cytoplasm. The nuclear structure is made up of masses of chromatin which fade off gradually into the surrounding nuclear structure, giving a soft, cloud-like effect in contrast to the nuclear structure of thrombocytes and erythrocytes. In the cytoplasm of lymphocytes one finds large, coarse mitochondria as a constant feature. These Altmann bodies, which stain specifically with the Janus green, are apt to be perinuclear in their distribution and are sometimes grouped on one side of the nucleus. Many of the lymphocytes possess one or several small, red bodies in the cytoplasm. These structures stain a dark red in contrast to the brick-red of the monocyte granules and the brown of the thrombocyte segregation bodies. These neutral red bodies of the lymphocyte are round and are refractive, whereas the neutral red bodies of the monocytes and thrombocytes are non-refractive. The lymphocytes are only slightly motile in very fresh preparations. They move slowly with the nucleus near the front of the cell.

The accompanying table (Table I) gives the average total and per-

TABLE I

Blood Cell Counts of Normal Chickens

Fowl No.	Date	Total erythrocytes per cu. mm.	Total thrombocytes per cu. mm.	Total leucocytes per cu. mm.	Hemoglobin (Newcomer)	Eosinophiles with rods (per cent)	Pseudo-eosinophiles (per cent)	Basophiles (per cent)	Monocytes (per cent)	Lymphocytes (per cent)	Myelocytes (per cent)	Unclassified (per cent)	Total eosinophiles with rods per cu. mm.	Total pseudo-do-eosinophiles per cu. mm.	Total basophiles per cu. mm.	Total monocytes per cu. mm.	Total lymphocytes per cu. mm.
	11/ 2/27	3,270,000		17,670	65	31		10	14	42		1	6,008		1,767	2,474	7,421
	11/ 9/27	3,310,000	101,090	25,920	56	25	1	2	9	62		1	6,480	259	518	2,333	16,070
	11/12/27	3,220,000	46,137	11,830	60	26		3	10	60			3,076		355	1,183	7,098
	11/14/27	3,520,000	40,800	12,000	60	18	3	4	13	62	1		2,160	360	480	1,560	7,440
55 B	11/19/27	3,020,000	17,961	8,553	65	32		6	8	54			2,737		513	684	4,619
	11/21/27	3,600,000	13,793	13,793	65	23	3	3	18	52			3,172	414	414	2,483	7,172
	11/26/27	2,960,000	23,716	26,351	70	31		6	23	40		1	8,169		1,581	6,061	10,540
	11/28/27	2,300,000	29,716	11,607	70	25	1	2	15	57			2,902	116	232	1,740	6,616
	12/ 1/27	3,540,000	39,130	13,043	70	19	1	3	8	69			2,478	130	390	1,043	9,000
	12/ 6/27	3,660,000	52,800	28,225	68	23	1	7	19	50			6,492	282	1,976	5,363	14,113
64 B	4/ 7/28	4,620,000	9,420	15,710	60	49	1	5	19	26			7,700	157	786	2,985	4,085
	4/11/28	3,700,000	17,976	29,960	60	43	1	10	9	37			12,882	300	3,000	2,700	11,085
51 B	10/20/27	2,810,000	35,156	9,375	60	37	3		7	53			3,468	281		656	4,970
54 B	11/ 2/27	3,070,000	47,823	17,390	57	33	8	5	5	48			5,739	1,391	870	870	8,347
65 B	4/ 7/28	3,760,000	23,280	23,280	75	66		1	13	20			15,365		233	3,026	4,656
	4/11/28	3,620,000	20,882	20,882	70	48	11	5	21	15			10,023	2,297	1,044	4,385	3,132
66 B	4/11/28	3,600,000	5,408	6,760	77	44	2	1	14	39			2,974	135	68	946	2,636
	4/16/28	3,390,000	25,025	22,750	72	24	2	5	8	61			5,460	455	1,137	1,820	13,878
1 C	10/19/28	3,750,000	10,672	26,686	46	48	1	1	17	32			12,809	267	267	4,536	8,540

10/22/28:															
11:45	2,810,000	52,382	33,795	52	33	2	3	34	28		11,152	676	1,014	11,490	9,463
2:15	2,835,000	30,640	30,640	48	32		2	32	34		9,805		613	9,805	10,417
2:45	2,730,000	28,192	26,597		40	4	2	20	34		10,639	1,064	532	5,319	9,043
2 C	3:15	3,030,000	34,513	33,508		31	2	7	23	37		10,387	670	2,345	7,708	12,398
3:45	3,080,000	21,912	27,390		44		4	25	27		12,052		1,096	6,847	7,395
4:30	2,830,000	42,543	39,030		30	3	7	30	29	1	11,709	1,171	2,732	11,709	11,319
10/23/28	2,970,000	11,556	29,630	52	38		2	38	19	3	11,259		593	11,259	5,630
4 C	10/29/28	2,780,000	27,960	41,730	58	46		4	24	26		19,196		1,669	10,015	10,850
5 C	1/24/29	3,610,000	142,048	73,600	57	29	1	2	3	65		21,344	736	1,472	2,208	47,840
6 C	1/25/29	3,150,000	27,306	33,300	69	39		10	17	34		12,987		3,330	5,661	11,322
Average	3,267,000	34,990	24,586	62.9	34.72	1.76	4.21	17.1	41.79		8,642	385	1,069	4,444	9,900

centage values of each of the structural elements found in the blood of eleven domestic fowls. The total number of counts reported is 29. The average red blood cell count was 3,267,000 cells per cubic millimeter. The highest and lowest counts were 3,760,000 and 2,300,000 erythrocytes per cubic millimeter respectively. The average thrombocyte value was 34,990 cells per cubic millimeter, with a variation from 5,408 to 142,048 thrombocytes per cubic millimeter. Total leucocytes averaged 24,586 cells per cubic millimeter and varied between 6,760 and 73,600 cells per cubic millimeter. The hemoglobin (Newcomer method) varied from 46 per cent to 77 per cent. The average was 62.9 per cent.

Eosinophiles with rods varied between 18 and 66 per cent in different animals and averaged 34.72 per cent. Pseudo-eosinophiles with small, round granules were not encountered in ten of the counts. The highest percentage obtained was 11, and the average 1.76 per cent. Basophiles were found in all except one animal. The highest percentage value obtained was in Rooster 64, where they were 10 per cent. The average value was 4.21 per cent. Monocytes were found to be high in the chicken, varying from 3 to 38 per cent and averaging 17.1 per cent. Lymphocytes were usually more numerous than any of the white blood cells except thrombocytes. They also showed a wide variation between 15 and 69 per cent. The average value was 41.79 per cent. Myelocytes were rarely encountered and unclassified cells were few in number.

The total numbers per cubic millimeter of each of the types of leucocytes are given in Table I. Their average values obtained were:

	per cubic millimeter
Eosinophiles with rods	8,642
Pseudo-eosinophiles	385
Basophiles	1,069
Monocytes	4,444
Lymphocytes	9,900

Discussion of Blood Cells

The literature records the observation of numerous investigators on the blood of the fowl. Some of the more important of these studies have been summarized in Table II.

TABLE II

Blood Counts of Domestic Fowls as Recorded in Literature

Authors	Erythrocytes per cu. mm.	Leucocytes per cu. mm.	Thrombocytes per cu. mm.	Eosinophiles with rods	Pseudo-eosinophiles	Basophiles	Monocytes	Lymphocytes Large	Lymphocytes Small	Hemoglobin	Degenerating types	Unclassified	Clasmatocytes
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Stölzing (9)	3,860,000												
Malassez (10)	3,100,000												
Hayem (11)	2,400,000	26,300											
Moore (12)	3,637,000	20,081											
Ward (13)	3,283,000	36,185											
Mack (14)	3,017,000	55,272								87.3			
Albertoni and Mazzoni (4)	2,460,000	32,300	45,566	29.5	4.5	2.2	None	63.8		62.0			
Klieneberger and Carl (15)	3,117,000	35,000 to 60,000	22,900 to 130,000										
Blain (2)	2,000,000 to 3,000,000		None	49.4	8.7	3.6	5.7	35.5			16.5		
Warthin (16)		18,630 to 12,000		21.5	10.0	2.0	None	32.8	14.5				
Burnett (17)	3,324,000	17,921		28.8	3.3	4.3	5.5	58.0		76.0			
Schmeisser (18)	2,500,000 to 4,500,000	20,000 to 80,000		29.6	4.3	2.2	19.4	42.3		45.0 to 75.0 (Sahli)		2.2	
Breusch (19)	3,463,000	33,300		17.7	4.1	2.5	9.2	66.5					
This study (average values)	3,267,000	24,586	34,990	34.7	1.8	4.2	17.0	41.8		62.9 (Newcomer)	Rare	Rare	Rare

It will be seen that Mack (14) records 55,272 leucocytes per cubic millimeter as the normal count, whereas Blain's (2) and Burnett's (17) data show 18,000 cells per cubic millimeter as the average normal value. Likewise the thrombocytes have been variously recorded. Most of the investigators have failed to enumerate them. Klieneberger and Carl (15) state that they vary from 22,000 to 130,000 per cubic millimeter. On the other hand, Blain (2) has failed to find any of these structures. The percentages of the types of leucocytes have yielded markedly different figures in the hands of different investigators. Eosinophiles with rods are reported by Breusch (19) as constituting 17.7 per cent of the total leucocytes, whereas Blain (2) found 49.4 per cent of these cells. Klieneberger and Carl (15) identified no monocytes or transitional cells in normal domestic fowls. On the other hand, Schmeisser (18) states that there are 19.4 per cent of these elements in the blood.

An analysis of the literature reveals four causes for most of the discrepancies. These causes are: study of insufficient numbers of animals, failure to make a sufficient number of observations on single animals, lack of an adequate method of counting white blood cells, and confusion of thrombocytes with other elements of the blood. It must also be borne in mind that there are large individual fluctuations of the leucocyte counts of fowls which are considerably greater than those encountered in mammals.

Still another factor which has led to confusion in the interpretation of blood cells of the fowl is the method of classification of the granular leucocytes.

Niegolewsky (20), for example, described two types of oxyphil granular cells, one with large refractive granules and another with fine oxyphil granules. The former were numerous and the latter few in number. In addition he found two types of basophiles and also neutrophilic leucocytes. The latter had round or polymorphous nuclei and fine granules. Grünberg (21) described two types of eosinophilic leucocytes: (a) cells with crystalloid granules, and (b) cells with globular-like granules. These latter he stated were similar to the polymorphonuclear cells of man. Hirschfeld-Kaszmann (22) stated that three types of eosinophilic granular cells occurred: (a) cells with segmented nuclei and crystalloid granules, (b) cells with single nuclei and pale rod-shaped granules, and (c) cells similar to (b) but having round granules. They found no neutrophilic granular cells. Burnett (17) did not call the polymorphonuclear cells with rod or spindle-shaped granules eosinophiles. He believed that these cells resembled in their staining reaction and biological properties the polymorphonuclear neutrophiles of mammals. He found eosinophilic leucocytes with round granules which were similar to the eosinophiles of mammals. Kasarinoff (23) described the same types

of eosinophiles as did Grünberg (21). Klieneberger and Carl (15) found two types of eosinophiles. Those with rod-shaped granules they called pseudo-eosinophiles and those with small granules they labelled as eosinophiles with small granules.

Descriptions of Bone Marrow Cells

The method which has been applied in these studies to the bone marrow has been a modification of that employed for blood and is the same as that which has been used by Sabin and Doan (24) and others for the study of bone marrow, lymph nodes, and other tissues of animals. Neutral red and Janus green films are prepared as for making supravital studies on blood, except that the concentrations of the dyes are increased about three fold. Small pieces of representative bone marrow are then removed from an anesthetized or freshly killed animal. A suspension is made on the prepared slide by mixing the small piece of marrow in a drop of normal saline, or better yet, with the animal's own serum. This material is spread out on the slide over an area the size of the coverslip. The coverglass is then allowed to fall gently on the prepared marrow. The preparation is quickly rimmed with vaseline or melted paraffin and can be studied immediately in a constant temperature box at about 38 or 39°C. Material prepared in this way gives a fairly uniform distribution of the cells and a thin enough film so that the individual cells can be studied and counted. The cells survive for hours if kept at body temperature.

A complete review of the histology of the bone marrow has recently been written by Sabin (25). For this reason it is unnecessary to review again the literature on this organ.

The types of cells found in the bone marrow of the chicken are, in addition to the fully matured formed elements of the blood, immature forms of all the leucocytes and red blood corpuscles. The relative numbers of the various types of cells are recorded in Table III. In this table the numerical values are given of the cells found in the bone marrow of four normal, Plymouth Rock roosters, about 8 months of age.

The cells present in largest numbers in films of the bone marrow are mature red blood corpuscles. They constitute an average of 53.8 per cent of all the cells present. Mature polymorphonuclear eosinophiles, on the other hand, constitute only 3.9 per cent of all the cells and 8.18

TABLE III

Survey of Cells of Bone Marrow of the Domestic Fowl

Rooster No.	Marrows examined	Total number of cells counted			Mature R.B.C. Per cent of all cells	Eosinophiles								Neutrophiles			
		Mature R.B.C.	Mature P.M.E.	All other cells		Mature P.M.E.		Myelocytes "A"		Myelocytes "B"		Myelocytes "C"		Mature P.M.N.		Myelocytes	
						Per cent of all cells	Per cent of cells exclusive of mature R.B.C.	Per cent of all cells	Per cent of cells exclusive of mature R.B.C.	Per cent of all cells	Per cent of cells exclusive of mature R.B.C.	Per cent of all cells	Per cent of cells exclusive of mature R.B.C.	Per cent of all cells	Per cent of cells exclusive of mature R.B.C.	Per cent of all cells	Per cent of cells exclusive of mature R.B.C.
2 C	Rt. femur / Lt. " / Rt. tibia	769	80	798	46.86	4.85	9.11	0.48	0.91	2.31	4.33	8.74	16.40			0.48	0.91
1 C	Rt. femur / Lt. " / Rt. tibia " / Lt. "	839	49	375	66.43	2.69	8.02			0.95	2.83	8.16	24.29			0.24	0.71
4 C	Rt. femur / Rt. tibia / Lt. " / Rt. radius	828	12	660	55.20	0.80	1.79	0.47	1.04	2.20	4.91	9.93	22.17			0.13	0.29
5 C	Rt. femur / Lt. " / Rt. tibia / Lt. "	876	138	862	46.70	7.41	13.80	0.75	1.40	1.17	2.20	11.30	21.2	0.16	0.30	0.21	0.40
Average........		828	70	674	53.8	3.94	8.18	0.42	0.84	1.66	3.57	9.50	21.02	0.04	0.08	0.26	0.58

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TABLE III—*Concluded*

Rooster No.	Marrows examined	Basophiles		Monocytes		Small, round cells, probably lymphocytes		Undifferentiated cells—megalo-, lympho-, mono-, myeloblasts		Erythroblasts		Clasmatocytes		Osteoclasts		Unclassified	
		Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.	Per cent of all cells	Per cent of cells exclusive of mature R. B. C.
2 C	Rt. femur Lt. " Rt. tibia	0.31	0.58	0.67	1.25	13.78	25.85	4.37	8.09	16.15	30.29	1.09	2.05			0.12	0.23
1 C	Rt. femur Lt. " Rt. tibia Lt. "					6.65	19.81	7.67	22.87	7.05	21.00					0.16	0.47
4 C	Rt. femur Rt. tibia " Lt. " Rt. radius			5.93	13.24	10.93	24.40	2.74	6.11	10.13	22.62	0.48	1.64	0.13	0.30	0.67	1.49
5 C	Rt. femur Lt. " Rt. tibia Lt. "	0.05	0.10	3.09	5.80	14.71	27.60	3.04	5.70	11.46	21.5	0.39	0.92	0.03	0.07	0.24	0.55
	Average.........	0.09	0.17	2.42	5.07	11.52	24.41	4.46	10.69	11.20	23.85						

per cent of all the cells, exclusive of mature red blood corpuscles. The accompanying table has been arranged to give the percentage value of each constituent element both in relation to the total of all the cells present and in relation to the number of cells, exclusive of mature red blood corpuscles. The reason for this is that most of the mature erythrocytes probably represent extravasations from traumatic hemorrhage and are not true parenchymal elements of the marrow.

Myelocytes (Figs. 14, 15, 16).—In this paper the myelocytes have been grouped arbitrarily into A, B, and C classes, according to the plan employed by Sabin, Austrian, Cunningham, and Doan (26). Myelocytes A are of the earliest type and represent the next stage of development after the myeloblast. Myelocytes A include cells with 1 to 10 specific granules in the cytoplasm. Myelocytes B (Figs. 14, 15) have more specific granules than Myelocytes A, but do not possess their full quota. Myelocytes C (Fig. 16) represent the next stage of development in which there is the full quota of specific granules in the cytoplasm, but the cells still retain their mononuclear characteristics.

The myelocytes of the chicken's blood are not only distinguished by the number of their specific granules, but also by their character. The granules are almost always round or globular, whereas the fully mature cell possesses elongated, rod-shaped granules. Moreover, the younger the specific granules are the more intensely they stain with neutral red. The mature granules of the adult polymorphonuclear cell and of the very late myelocytes stain a yellowish copper color with neutral red. The earlier myelocyte granules (Fig. 15) are darker in color, staining a brownish red. Mitochondria are readily seen in the early myelocytes. They are scattered through the cytoplasm and are delicate rods or dots which stain specifically with Janus green. With greater accumulation of specific granules, the mitochondria become less conspicuous and sometimes cannot be seen. Myelocytes A are are few in number in normal bone marrow. The average value was slightly less than 1 per cent of the cells, exclusive of mature erythrocytes. Myelocytes B composed 3.57 per cent. These cells were present in about the same relative proportions as Sabin and Doan (24) found them in normal rabbit bone marrow. Myelocytes C were represented by 21.02 per cent of the cells, distinctly less than their proportion in rabbit bone marrow.

Pseudo-eosinophilic myelocytes and polymorphonuclear pseudo-eosinophiles were only occasionally found in the bone marrow, where they constituted one-half of 1 per cent of the cells. They were identical with the pseudo-eosinophilic cells of the blood.

Basophiles.—These cells were likewise rare in the bone marrow. They, too, were identical with the basophilic cells which were found in the blood and have been described in an earlier part of this paper. In two of the animals no basophiles were seen in the marrow films. The average value for basophiles in the bone marrow was less than 0.2 per cent. Sabin and Doan (24) found in rabbits that they constituted from 0.29 to 2.02 per cent of the bone marrow cells.

Monocytes.—About 5 per cent of the parenchymal elements of the marrow are monocytes. They are identified by the criteria previously given in the section on blood cells, and need no further description here. It is not difficult to distinguish them from the early myelocytes because of the larger and more refractive granules of the latter.

Lymphocytes.—Contrary to the findings in bone marrow of mammals, there is a considerable number of small round cells (Fig. 3) in every film of bone marrow equivalent to about 25 per cent of the cells. They are probably small lymphocytes. It is often difficult in the marrow to differentiate these elements from primitive cells and thrombocytes. The characteristics which render the distinction easy in a thin film of blood are often obscured by the crowding of the cells and lack of perfectly uniform staining.

Undifferentiated Cells (Fig. 13).—The early undifferentiated cells of the bone marrow I have chosen to group together under one heading for the reason that I have not been able to say in numerous instances whether given cells were megaloblasts, monoblasts, lymphoblasts, myeloblasts, or primitive cells. The problem of sub-classifying these "blast" cells in the marrow of the chicken is much more difficult than to recognize and classify them when they occur in the blood. The reasons are obvious. The staining is not as uniform, the cells are more crowded, and one cannot have as an aid the character of the other accompanying cells, which aid is often of great value in a study of blood films. In the bone marrow, spleen, and lymph nodes of mammals it is usually not difficult to segregate the cells into the various types of blasts, but I do not believe that there is sufficient evidence in the

chicken for such a rigid sub-division of the undifferentiated marrow cells. This group of blast cells represents about 10.5 per cent of the parenchymal elements. They are about the size of myelocytes. The nucleus may be central or peripheral and frequently contains a large nucleolus. The cytoplasm usually contains no stainable material in supravital films.

Erythroblasts (Fig. 12).—Immature red blood cells are separated from the mature red corpuscles by their larger and more conspicuous nuclei, by the presence of more neutral red bodies in the cytoplasm, and often by a decreased amount of hemoglobin. The immature red corpuscles are frequently less oval and more round than the adult forms. The total number of erythroblasts represents about 24 per cent of the cells, exclusive of mature red blood cells, and about 11 per cent of the total cells present.

Clasmatocytes (Macrophages).—The characteristic by which these cells are recognized is dependent largely on their phagocytic properties. They engulf leucocytes, red corpuscles, and débris. The vacuoles of the living cells stain brilliantly with various shades of red, yellow, and brown. These vacuoles increase in size with longer exposure to the dye, and are not arranged in any definite pattern. The nucleus is usually round and placed near the center of the cell. The shape of macrophages is often irregular. They vary in size, but are commonly about the size of monocytes or other large leucocytes. These cells constitute about 1 per cent of the marrow elements.

Osteoclasts (Fig. 17).—The surveys of the bone marrows of four chickens reported in this communication show osteoclasts to be present in only one. In this animal they represented 0.3 per cent of the marrow cells. The ends of the long bones often contain many spicules of bone, and in these regions osteoclasts are more numerous than in the shafts. They are very large, multinucleated cells. The nuclei do not appear to have any definite pattern, but are of about equal size and scattered through the cytoplasm. The cytoplasm often contains considerable numbers of vacuoles or granules which are quite homogeneously distributed throughout the cytoplasm and may tend to obscure the nuclei. These vacuoles stain brown with neutral red.

Thrombocytes have not been observed in the marrow of the long

bones of these chickens. Their origin is obscure and work is now in progress to determine more about the life history of these elements.

SUMMARY AND CONCLUSIONS

1. A simple, direct method of counting leucocytes of the fowl is described.

2. Twenty-nine complete, morphological studies of the blood of eleven domestic fowls are recorded.

3. The characteristics of the cells found in the blood and bone marrow are described in detail and their relative numbers reported.

4. The supravital technique, using neutral red and Janus green, enables one to separate and classify accurately the confusing cells of the blood and bone marrow.

5. These studies provide a basis for future experimental studies on the blood and bone marrow cells of the fowl.

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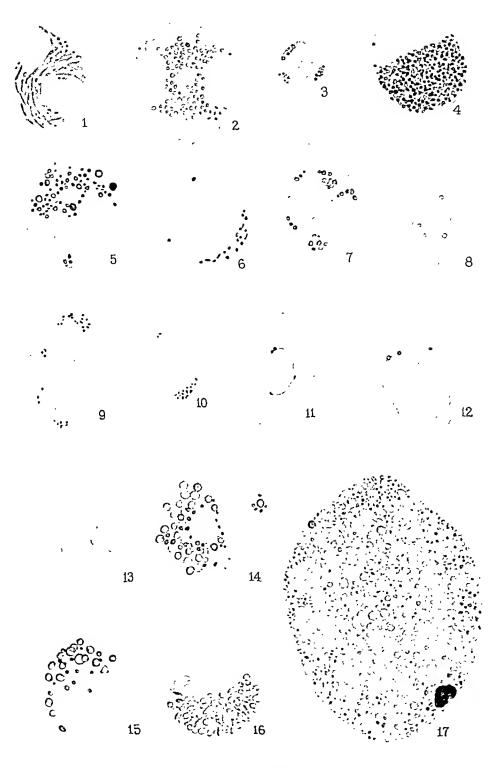
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EXPLANATION OF PLATE 6

FIG. 1. Typical polymorphonuclear eosinophile with rods from peripheral blood. Stained with neutral red.

FIG. 2. Pseudo-eosinophile from peripheral blood of domestic fowl. This cell is slightly larger than the typical pseudo-eosinophile. Stained with neutral red and Janus green.

FIG. 3. Small, round cell of bone marrow of the domestic fowl, probably a lymphocyte.

FIG. 4. Basophilic leucocyte of the peripheral blood. Note that the granules vary somewhat in their affinity for neutral red.

FIG. 5. Monocyte of the peripheral blood. Mitochondria stained with Janus green and segregation bodies stained with neutral red. Note the tendency for grouping of neutral red bodies about the centrosphere, a common, but not invariable characteristic of these cells.

FIG. 6. Large lymphocyte from peripheral blood. Stained with neutral red and Janus green. There were no neutral red bodies in this cell, a fairly common finding. Mitochondria are large.

FIG. 7. Two thrombocytes grouped together in the peripheral blood. No mitochondria were seen in these cells, but there were numerous neutral red vacuoles. Stained with neutral red and Janus green.

FIG. 8. A degenerating red blood corpuscle of peripheral blood. Note the absence of hemoglobin, but the preservation of the cellular membrane. A few refractive, unstained bodies are near or on the nucleus. These cells have been confused with thrombocytes. Compare with Figs. 7 and 9.

FIG. 9. Two thrombocytes of peripheral blood. Mitochondria are more conspicuous than in the usual thrombocyte. A neutral red vacuole has begun to develop. Stained with neutral red and Janus green.

FIG. 10. Intermediate lymphocyte of peripheral blood. Slightly larger than a small lymphocyte. Stained with neutral red and Janus green. Two small neutral red bodies are present.

FIG. 11. Typical erythrocyte of peripheral blood.

FIG. 12. Erythroblast from the bone marrow. Note the smaller amount of hemoglobin than in the mature erythrocyte.

FIG. 13. An undifferentiated cell of the bone marrow. This cell has an eccentric nucleus. Many of them have nuclei in the center of the cell. Neutral red and Janus green stain.

FIG. 14. A myelocyte "B" of the bone marrow. Specific granules are refractive and round. Stained with neutral red. Mitochondria are often abundant, but are not shown in this drawing.

FIG. 15. Myelocyte "B" of a somewhat earlier stage than that shown in Fig. 14. Specific granules vary considerably in size and take a somewhat deeper color than in the later myelocytes. Stained with neutral red.

FIG. 16. Myelocyte "C" of bone marrow. Some of the specific granules are beginning to assume the color and shape of those of the fully mature polymorphonuclear eosinophile shown in Fig. 1. Neutral red stain.

FIG. 17. Osteoclast of the bone marrow. Nuclei are round and almost obscured by the neutral red bodies.

(Forkner: Blood and bone marrow cells of domestic fowl)

THE RÔLE OF CARBOHYDRATES IN BIOLOGICAL OXIDA-
TIONS AND REDUCTIONS. EXPERIMENTS
WITH PNEUMOCOCCUS

RENÉ DUBOS, Ph.D.

INTRODUCTION

When repeatedly washed in saline solution, living cells lose in a large measure their ability to oxidize and to reduce. This fact was established by studies of oxygen consumption and methylene blue reduction with muscle tissues and yeasts. It led to the concept that the oxidation-reduction properties of a tissue or a cell are really due to the oxidation-reduction properties of certain metabolites after they have become "activated" by the tissue or cell under consideration.

The analysis of phenomena of this nature in bacterial systems was apparently begun by Harden and Zilva (1) in 1915. They found that washed cells of *B. coli* which reduce methylene blue only very slowly, reduce the same dye very rapidly in the presence of various substances. This work was much extended by Quastel and his associates (2) who referred to the washed cells as "resting bacteria." "Resting bacteria are simply bacteria in a state of non-proliferation and may be investigated in a manner similar to enzyme or catalytic systems." Working with *B. coli*, *B. pyocyaneus* and *B. alkaligenes*, they found that these organisms can activate the reduction of methylene blue by certain sugars, amino acids, and fatty acids (especially formic, lactic and succinic). *B. coli* was found to have an even wider range of activating action than muscle, 56 substances out of 103 studied being "activated" in this manner. Quastel (3) suggested that the process of "activation" consists in a polarization of the substrate molecules at particular regions or centers on the surface structures (interfaces) of the cell

In 1914, Cole (4) investigated the mechanism of the reaction whereby Pneumococci transform hemoglobin into methemoglobin. The reaction does not occur when hemoglobin is added to an emulsion of washed cells in salt solution. However, if minute traces of dextrose (or a number of other organic substances) be added to such a mixture, the reaction quickly occurs; it is always conditioned by the presence of oxygen. Avery and Neill (5) have described a large number of other oxidation-reduction processes exhibited by Pneumococcus cultures; amongst

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which we shall consider in our studies the reduction of methylene blue and the formation of peroxide. The fact that these processes have the same optimum and limiting condition (temperature, pH, etc.,) suggests that they are brought about by one and the same system. They are exhibited not only by the whole intact cells, but also by sterile extracts of these cells prepared under proper conditions. They can also proceed under conditions of pH and temperature which do not permit active growth. As in the case of muscle, yeast and *B. coli*, the washed cells of Pneumococcus are unable to reduce, to consume oxygen, or to form peroxide. However, the oxidative-reductive properties can be restored by the addition to the washed cells of the cell washings, and of aqueous or alcoholic extracts of muscle, yeast and vegetable tissues. The oxidation-reduction system of the Pneumococcus cell therefore appears to consist of at least two components: 1) the heat stable component, just referred to, which can be readily washed out of the cell and is not necessarily of Pneumococcus origin. 2) a labile cellular component, not removed by washing, inactivated by 10 minutes heating at 65°C.

In the experiments to be presented here, we have attempted to analyze further the mechanism of bacterial oxidations and reductions and to establish new reactions of physiological significance. This report is limited to the influence of carbohydrates, especially glucose, as these substances appear to be of primary importance in the phenomena of growth. Although most of the work has been done with Pneumococcus cells, it is hoped that the facts established will prove to be of a more general significance in an understanding of cellular physiology.

EXPERIMENTAL

Experimental Methods

1. Bacteriological.—The experiments have been carried out with 3 strains of Pneumococcus: Type I (1/219/4), Type III (A/66/73) and an R cell derived from Type II (D/39/R). The results obtained apply to all 3 strains; for the sake of simplicity, all protocols refer to strain D/39/R.

Unless otherwise stated, very young cultures (6 hours old) were used in the tests; these cultures were obtained by seeding 2 cc. of a young culture into 150 cc. of meat infusion broth containing 1 per cent Witte's peptone and 0.03 per cent dextrose. The cells were separated from the medium and washed in physiological salt solution by centrifugalization (30 minutes—about 3000 rotations per minute).

By "sugar free meat infusion," we understand beef infusion prepared according to the standard method and from which the sugars have been removed by growing Pneumococcus in the infusion and filtering out the cells.

2. Chemical.—All the tests were performed in phosphate buffer solutions (0.03 molar) at pH 8.0.

The dye solutions were prepared as described in an earlier paper (6).

The following substances were used as source of oxidized thiol groups; a) glutathione (in the form of meat infusion); b) saturated cystine solution at pH 8.0; c) a product obtained by the auto-oxidation of thioglycollic acid (Eastman) in the presence of air, with sodium nitroprusside as a catalyst (in dilute ammonia solution); we shall refer to the last product as "oxidized thioglycollic acid" without attaching to this expression any real meaning concerning the exact nature of the compound.

The reduction experiments were carried out in Noguchi tubes under a vaseline seal of 2 inches thickness, at room temperature (about 20°C.). The nitroprusside test was used to detect the presence of reduced thiol groups. The formation of peroxide was tested by transferring 10 cc. of the systems under consideration to 150 cc. Erlenmeyer flasks which were incubated at room temperature; the titanium sulphate test was used for detecting the peroxide present.

TABLE I

Reduction of Methylene Blue by Pneumococcus Cultures at Different Periods of Growth

No. of hours after inoculation at which samples were taken	Time required for the reduction of 0.2 cc. of 0.0025 M methylene blue by 5 cc. culture	
	Plain broth culture	Glucose broth culture
1 hour	6 hours	6 hours
3 hours	4 "	3 "
4 " 30 minutes	2 " 20 minutes	1 " 5 minutes
6 "	2 " 10 "	0 " 30 "
7 " 30 minutes	4 "	0 " 25 "
12 "	8 "	0 " 30 "
24 "	18 "	2 " 10 "

The Reduction of Methylene Blue by Pneumococcus Cultures

When one attempts to determine the "reducing power" of a Pneumococcus culture, it is found that even under the same conditions of temperature and reaction, the rate of reduction of methylene blue varies a great deal, according to the composition of the medium and the age of the culture.

Experiment 1.—Two flasks, each containing 150 cc. of plain broth, were seeded with 2 cc. of a young culture of Pneumococcus. One of the flasks received in addition 0.1 per cent glucose. The two flasks were incubated at 37°C. and samples were removed after 1, 3, 4½, 6, 7½, 12 and 24 hours incubation. The reaction of these

samples was adjusted to pH 8.0 with NaOH and a reduction test was set up with 0.2 cc. of 0.025 M methylene blue. The times required for reduction are given in Table I.

Although no bacterial counts were made, the turbidity of the cultures indicated that the maximum growth was obtained after 6–8 hours incubation. The fact that during the early periods of incubation, the dye was more rapidly reduced in the glucose broth culture than in the plain broth culture may be accounted for by the more rapid multiplication of the organisms in the former medium. But the rapid decrease of the "reduction power" of the plain broth culture as compared with its constancy in the glucose broth culture cannot be attributed to the difference in the number of cells, for it is known that the number of R cells of Pneumococcus remain constant for at least 24 hours in plain broth cultures, while the same organisms begin to die off more rapidly in glucose broth. It appeared, therefore, that the constituents of the medium played an important rôle in the reducing power of the culture. This is shown by Experiment 2.

Experiment 2.—Flasks containing 150 cc. of plain broth were seeded with 1.25 cc. of a young D/39/R culture. 10 cc. samples were taken at different times after inoculation, and adjusted to pH 8.0. In each case, 2 samples received 0.1 cc. of a 1 per cent glucose solution and 2 were kept as control, one of the duplicate tubes in each series was placed in a bath of boiling water for 2 minutes. 2 cc. of 0.0025 M methylene blue was then added to each one of the tubes. The time required for reduction of the dye is given in Table II.

A number of conclusions can be drawn from this experiment: a) the addition of small amounts of glucose to Pneumococcus cultures brings about a more rapid reduction of the methylene blue; b) the decrease in the reduction power of a plain broth culture after the sixth hour of growth is probably due to the disappearance of the small amount of sugar originally present in the broth; c) the ability of the glucose to reduce methylene blue is probably due to some cellular factor, since it reaches its maximum at the time when the growth is maximum; d) this factor slowly loses its power as the culture ages.

It is known that oxidized methylene blue is bacteriostatic for Pneumococcus (7); this and the fact that the reduction of the dye occurs so rapidly (12 minutes) is an indication that the phenomenon of reduction

is not associated with a proliferation of the cells. The experiments with washed cells serve to demonstrate this point.

TABLE II

The Effect of Heating, Age of the Culture, and Addition of 0.1 cc. of 1 Per Cent Glucose on the Velocity of Reduction of 0.2 cc. of 0.0025 M Methylene Blue by Pneumococcus Cultures (10 cc.)

No. of hours after inoculation at which sample was taken		No. of cells per cc. of culture	Time required for reduction by:				
			Culture	Heated culture	Culture + dextrose	Heated culture + dextrose	0.1 cc. of 1 per cent dextrose
1 hour		10^7	12 hrs.	—*	12 hrs.	—	—
2 hours		10^9	9 hrs.	—	9 hrs.	—	
3 "	30 minutes	10^9	2 hrs. 15 min.	—	2 hrs. 15 min.	—	
5 "		10^9	55 min.	—	45 min.	—	
6 "	30 minutes	10^9	12 min.	—	10 min.	—	
8 "		10^{10}†	44 min.	—	13 min.	—	
10 "		10^{10}†	2 hrs. 30 min.	—	15 min.	—	
12 "	30 minutes	10^{10}†	6 hrs.	—	17 min.	—	
23 "		10^9	16 hrs.	—	27 min.	—	
26 "		10^9	—	—	45 min.	—	
30 "		10^9	—	—	48 min.	—	
47 "		10^6	—	—	6 hrs.	—	

* The sign — indicates that the dye was not reduced after 24 hours. The heated cultures, however, showed some reduction at that time. This reduction was of the same order as that exhibited by sterile media (6) and need not interest us here.

† The apparent increase in numbers observed after the 8th hour of incubation is probably due to the breaking up of the Pneumococcus chains

The Reduction of Methylene Blue by Washed Cells of Pneumococcus; Its Activation by Meat Infusion

Experiment 3.—A young culture of D/39/R in plain broth was centrifuglized, and the cells washed 7 times in saline. A sample corresponding to 15 cc. of culture was taken following each washing, and the ability of the washed cells to reduce methylene blue was tested in the absence and in the presence of glucose.

This experiment demonstrates that cells washed free of metabolites are unable to reduce methylene blue; however, they do reduce it rapidly

in the presence of glucose; the partial reduction obtained with the unwashed cells without further addition of sugar is to be traced to the metabolites associated with the cells and which are removed by repeated washings.   It is also evident that, as the washings are repeated, the cells progressively lose their ability to reduce in the presence of

TABLE III

The Reduction of Methylene Blue (0.1 cc. of 0.005 M) by Washed Cells of Pneumococcus

No. of washings	Time required for complete reduction	
	No glucose	0.2 cc. of 0.005 M glucose
0†	—*	2 minutes
1†	—	5 "
2	—	9 "
3	—	16 "
4	—	29 "
5	—	40 "
6	—	—
7	—	—

* The sign — indicates that the dye was not reduced in 24 hours.

† The first and second samples of cells showed a partial reduction of the dye in 24 hours, even without glucose.

TABLE IV

Influence of Sugar-free Meat Infusion on the Velocity of Reduction of Methylene Blue by Washed Cells of Pneumococcus in the Presence of Glucose

Washed cells	Methylene blue 0.002 M	Glucose 0.002 M	Meat infusion	Time required for complete reduction
cc.	cc.	cc.	cc.	
10	1	1		90 minutes
10	1	1	0.5	40 "
10	1	0	0 5	Reduction only partial after 24 hours

glucose.   Now, it had been observed in the course of previous experiments that the presence of sugar-free meat infusion increases the velocity of reduction of methylene blue by the Pneumococcus-glucose system, although a system containing washed cells and sugar-free meat infusion is unable by itself to bring about any *rapid* reduction.   An example of such an action is given in Experiment 4.

Experiment 4.—A young plain broth culture of Pneumococcus was centrifugalized and the cells washed twice in saline. The washed cells were suspended in buffer pH 8.0 and their reducing power tested with 1 cc. of 0.002 M methylene blue and 1 cc. of 0.002 M glucose—with and without the addition of 0.5 cc. sugar-free meat infusion. A tube containing 10 cc. of cells + 0.5 cc. meat infusion + 1 cc. of 0.002 M methylene blue, but no glucose, was used as control (Table IV).

Since sugar-free meat infusion is found to increase the velocity of reduction of methylene blue by a system containing Pneumococcus + glucose, it appeared possible that the action of the cell on the glucose might depend on two factors, one which cannot be removed by washing and another present in meat infusion. Experiment 5 bears on this point.

TABLE V

Influence of Washing the Cells and of the Addition of Meat Infusion on the Reduction of Methylene Blue by the Pneumococcus-Glucose System

No. of washings	No meat infusion	0.5 cc. of meat infusion
1	+*	+
2	+	+
3	+	+
4	—*	+
5	—	+
6	—	+
7	—	—

* The signs + and — indicate that the methylene blue was, or was not completely reduced after 24 hours incubation.

Experiment 5.—Young cells of D/39/R were repeatedly washed in saline. At each one of the washings, experiments with amounts of cells corresponding to 10 cc. of culture were made to determine the reduction of 1 cc. of 0.001 M methylene blue by 1 cc. of 0.001 M glucose in the presence and in the absence of meat infusion (Table V).

The results of Experiment 5 confirm the view that in the mechanism responsible for the reduction of methylene blue in the presence of glucose there are at least two constituents involved; one present in meat infusion, readily washed out of the cells, and heat stable; the other persisting in the cell despite repeated washing but eventually lost as result of the process. It is not known as yet whether this sec-

ond factor is simply washed out, or destroyed following some injury caused to the cell in the process of washing. It has been found to be heat-labile and completely inactivated by 10 minutes exposure at 55°C.

Meat infusion is well known to contain reducing substances, and it is important to know to what extent methylene blue can be reduced by sugar-free meat infusion in the presence of Pneumococcus cells.

Experiment 6.—Young cells of D/39/R were washed four times in saline, and 5 cc. amounts were used to determine the comparative reduction of methylene blue in the presence of glucose, sugar-free meat infusion, and glucose + meat infusion. The details of the experiment are given in Table VI.

TABLE VI

The Activation of the Pneumococcus-Glucose System by Sugar-free Meat Infusion

Washed cells	Glucose 0.001 M	Meat infusion	Methylene blue (cc. of 0.001 M solutions)									
			0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
cc.	cc.	cc.										
5	0	0	±	—	—	—	—	—	—	—	—	—
0	1	0	—	—	—	—	—	—	—	—	—	—
0	0	5	—	—	—	—	—	—	—	—	—	—
5	1	0	+*	±*	—	—	—	—	—	—	—	—
5	0	5	+	+	±	—	—	—	—	—	—	—
5	1	5	+	+	+	+	+	+	+	+	+	+

* In this table the signs + and − indicate that the dye had or had not been completely reduced in 3 hours. ± indicates that the reduction was complete only after 24 hours.

The results of Experiment 6 demonstrate that the rapid reduction of methylene blue by a system containing washed cells + meat infusion + glucose cannot be accounted for by the reducing power only of any one of the three components of this system or even by any combination of two of these components. A rapid reduction of the dye requires the presence of all three of them.

The Existence of a Definite Ratio Between the Amounts of Glucose Used and of Methylene Blue Reduced

In the course of the reduction which has just been described, the methylene blue is reduced and the glucose oxidized. An attempt has

been made to determine the relative amounts of the two substances involved in the reaction. However, great difficulties have been encountered in obtaining a constant ratio, although in all instances the reduction of 1 mol of methylene blue required from 0.7 to 1 mol of glucose. The following experiment is a typical example of the results obtained in such determinations.

Experiment 7.—Young cells of D/39/R were washed once in saline. This was found sufficient to rid the cell of practically all the metabolites they contained.

TABLE VII

The Quantitative Relation Between the Amounts of Glucose Used and of Methylene Reduced

Amount of cells	Methylene blue (0.001 M solutions)	Time in minutes required for complete reduction of the dye in the presence of the following amounts of 0.001 M glucose solution										
		0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
cc.	*cc.*											
15.0	1	—*	—	—	—	—	—	—	—	360	45	60
10.0	1	—	—	—	—	—	—	—	240	—	330	120
5.0	1	—	—	—	—	—	—	—	—	—	—	120
2.5	1	—	—	—	—	—	—	—	—	—	—	145
15.0	0.1	—	60	x*	x	x	x	x	x	x	x	x
10.0	0.1	—	100	x	x	x	x	x	x	x	x	x
5.0	0.1	—	80	x	x	x	x	x	x	x	x	x
2.5	0.1	—	120	x	x	x	x	x	x	x	x	x

* The sign — indicates that the dye was not yet completely reduced after 12 hours incubation; x indicates that the test was not made.

Cells washed only once do not need meat infusion to be able to activate glucose. Varying amounts of these cells were used with varying concentration of glucose and of methylene blue for a reduction test. The details and results of the experiment are given in Table VII.

The results of this experiment demonstrate once again the reduction of methylene blue by Pneumococcus in the presence of glucose, and the lack of reduction in the absence of the sugar. It will be seen that, whereas 2.5 cc. of cells could reduce 1 cc. of the methylene blue solution in the presence of glucose, 15 cc. of the washed cells alone could not even reduce 0.1 cc. of the same dye solution. It seems true also that the ratio of glucose to methylene blue becomes narrower as larger

amounts of cells are used for the test; and this, as we have just seen, cannot be accounted for by the metabolites present in the cells. The change in the glucose-methylene blue ratio might conceivably be due to side-reactions which become manifest only when larger amounts of cells are used. In experiments not reported here, it has been established that the time required for the reduction of 1 cc. of 0.001 M methylene blue is not dependent upon the amount of glucose used, when this amount is larger than 1 cc. of 0.001 M solution. The fact that the reduction with 0.7, 0.8 and 0.9 cc. of the glucose solution takes

TABLE VIII

Time Required for the Reduction of rH Indicators by the Pneumococcus-Glucose System

Dye			Amount of cells			
Name	Concentration 0.01 M	rH	20 cc.	10 cc.	5 cc.	2.5 cc.
	cc.					
2 chloro indophenol..	0.1	21.8	Immediate reduction		6 minutes	30 minutes
1 naphthol-2 sulfonate indophenol...	0.1	18.1	"	"	10 "	30 "
Methylene blue.....	0.05	14.4	5 minutes	10 minutes	15 "	1 hour
Indigo tetrasulfonate.	0.1	12.5	16 hrs.	20 hrs.	2 days	2 days
Indigo trisulfonate...	0.1	11.3	2 days	8 days	Only partly reduced in 2 weeks	reduced in
Indigo disulfonate...	0.1	9.9	2 "	8 "	" "	" "

longer than with 1 cc. is another evidence that we are dealing with at least two different reactions.

In conclusion, it appears that, under the proper conditions, the reduction of one molecule of methylene blue requires one molecule of glucose.

The Reduction of the Indicators of Oxidation-reduction Potentials by the Pneumococcus-glucose System

The work of the past few years has indicated that many biological systems develop reducing potentials much lower than that of reduced

methylene blue. Experiment 8 demonstrates that when glucose is placed in the presence of Pneumococcus cells, dyes with a very low rH are also reduced.

Experiment 8.—A young culture of D/39/R was centrifugalized and the cell suspended in buffer at pH 7.8. Amounts of the suspension corresponding to 20-, 10-, 5-, and 2.5 cc. of the original culture were added to solutions of the rH indicators for a reduction test. Each one of the tubes received also 0.2 cc. of a 1 per cent glucose solution. The time required for the reduction of the dyes is given in Table VIII.

Table VIII shows: 1) that the time of reduction increases as the amount of cell decreases; 2) that the dyes are decolorized in the order of the electromotive series; 3) that all dyes tested were reduced.

TABLE IX

The Reduction of Thiol Groups by Glucose (0.2 cc. of 1 Per Cent Solution) in the Presence of Cells of Pneumococcus

Nature of the oxidized thiol compounds	Incubation period (hours)			
	24	48	72	96
Glutathione (5 cc. of meat infusion).............	±*	±	+	+
5 cc. of saturated solution of cystine.............	—*	—	±	+
1 cc. of 0.05 per cent oxidized thioglycollic acid.....	+*	++*	++	++

* In this table   — indicates a negative test for —SH.
                   ± indicates a doubtful test for —SH.
                   + indicates a positive test for —SH.
                   ++ indicates a strongly positive test for —SH.

The Reduction of Thiol Compounds by the Pneumococcus-Glucose System

In view of the importance of glutathione in cellular metabolism and of the fact that thiol compounds have been found to be an essential component of the media used for the growth of Pneumococcus,[1] an attempt has been made to find out whether oxidized thiol compounds can be reduced by a system consisting of Pneumococcus and glucose.

Experiment 9.—The set up of the experiment was the same as in Experiment 8, except that meat infusion, cystine and oxidized thioglycollic acid (see Experimen-

[1] Unpublished observation.

tal Methods) were used instead of the dyes. The systems were incubated for several days, and the presence of reduced thiol groups was tested for by the nitroprusside test.

Experiment 9 brings out the interesting fact that oxidized thiol compounds are reduced by glucose in the presence of Pneumococcus cells. This reduction could be obtained with as little as 2.5 cc. of cells.

The Formation of Peroxide by Washed Cells of Pneumococcus in the Presence of Glucose and Meat Infusion

Avery and Neill (5) have conclusively shown that Pneumococcus cells, when washed in saline, lose their ability to form peroxide, but regain it in the presence of yeast extract or meat infusion. Their results also indicate that the reduction of methylene blue and the formation of peroxide are controlled by the same mechanism. It was therefore tempting to see if peroxide formation could be obtained with the same system (washed cells + sugar-free meat infusion + glucose) which proved to be able to reduce methylene blue.

Experiment 10.—Young cells of D/39/R were repeatedly washed in saline and samples taken after the first, third, and fifth washings. Amounts of cells corresponding to 25 cc. of culture were suspended in 10 cc. of buffer at pH 7.8, and glucose and sugar-free meat infusion were added as described in Table X. The mixtures were transferred to 150 cc. Erlenmeyer flasks and incubated at room temperature. A test for peroxide was made after different periods of incubation, and the results corresponding to 18 hour periods are given in Table X.

It appears from the results of Experiment 10 that washed cells of Pneumococcus can form peroxide when placed in the presence of glucose and of sugar-free meat infusion under aerobic conditions. However, this property is completely lost after the cells have been washed 5 times. Peroxide formation and methylene blue reduction therefore appear as two similar reactions, in which oxygen and methylene blue respectively act as hydrogen acceptor.

The Activity of Carbon Compounds Other than Glucose in the Presence of Washed Cells of Pneumococcus

The reduction of the indophenols, of methylene blue and of the indigoes by washed cells of Pneumococcus, has been attempted in the

presence of inulin, lactose, saccharose, maltose, dextrose, levulose, mannose, arabinose, xylose, ribose, mannite and glycerine. It would be too long to report now the results of these studies. However, it may be said that the following substances have been found to be the most active (in order of velocity of reduction): levulose, dextrose, galactose, mannose, maltose, lactose. These same substances were also found to give rise to the formation of peroxide in the presence of washed cells of Pneumococcus under aerobic conditions; the test for peroxide in

TABLE X

The Formation of Peroxide by Washed Cells of Pneumococcus in the Presence of Glucose and Sugar-free Meat Infusion

Amount of cells	No. of washings	Glucose (20 per cent solution)	Meat infusion	Peroxide
cc.		cc.	cc.	
0		0.2	0.5	—
25	1	0	0	—
25	1	0.2	0	+
25	1	0	0.5	—
25	1	0.2	0.5	++
25	3	0	0	—
25	3	0.2	0	±
25	3	0	0.5	—
25	3	0.2	0.5	+
25	5	0	0	—
25	5	0.2	0	—
25	5	0	0.5	—
25	5	0.2	0.5	—

the same system was doubtful or negative when other compounds were used which bring about reduction of the methylene blue only slowly or not at all.

DISCUSSION

An interpretation of the facts which have just been presented requires an understanding of the nature of the interreaction between the glucose and the Pneumococcus cell. Is the glucose molecule split into reducing products, or does it itself become highly reactive owing to

some molecular rearrangement? The determination of the ratio between the amounts of glucose used and of methylene blue reduced is the first step in the elucidation of this problem.

As indicated above (Experiment 7), the value of this ratio seems to be affected by a number of side reactions, but a ratio very close to 1 can be obtained under proper conditions. It may be recalled here that Quastel has already attempted to determine the value of the same ratio. He found that, at pH 7.2, a single molecule of glucose donates at least 4 (and possibly 6) atoms of hydrogen to methylene blue in the presence of *B. coli*; galactose was less active and it was impossible to state whether this sugar donates more than 2 atoms of hydrogen per molecule. The least quantity of formic acid which could accomplish complete reduction of methylene blue was the amount to be expected if formic acid donated 2 atoms of hydrogen per molecule. Now, it is perhaps reasonable to assume that *B. coli* has a wider range of activation (in Quastel's terminology) than Pneumococcus, and that many of the products which result from the splitting of the glucose molecule by the cell become able to reduce methylene blue in the presence of *B. coli*, but not in the presence of Pneumococcus.[2] This would account for the narrow glucose- methylene blue ratio obtained with the organism first mentioned.

As a result of these observations, it appears likely that the first and fundamental reaction, in the reduction of methylene blue by the Pneumococcus-glucose system is one in which one molecule of the sugar becomes able to cause the reduction of one molecule of the dye, *i.e.*, to donate two atoms of hydrogen.

Nothing is known of the mechanism of the reaction. However, the following possibilities may be considered:

1. The glucose molecule is split into two fractions, each one donating one hydrogen atom.

2. The glucose molecule undergoes some rearrangement; two of its hydrogen atoms becoming "activiated." Examples of such "activation" are not unknown; succinic acid, for instance, although unable to reduce methylene blue by itself, does it in the presence of muscle tissue

[2] It has been established for instance that succinic acid, which is an excellent hydrogen donator in the presence of *B. coli* is inactive in the presence of Pneumococcus (unpublished observation).

and *B. coli* and is oxidized to fumaric acid in the course of the process. Concerning the hypothetical "activation" of glucose, it may be mentioned here that Aubel, Wurmser, Geloso, and Genevois (8) have observed the establishment of highly reducing potentials by glucose solutions at different electrodes. It would not be without bearing on this discussion to observe the potentials obtained with sterile glucose solutions compared with glucose in the presence of Pneumococcus cells.

3. Whatever may be the nature of the reducing substances formed from the glucose, another question remains to be answered. Do the glucose derivatives reduce the methylene blue directly, or do they act first on some of the cell components which in their turn reduce the hydrogen acceptor?

A knowledge of those constituents of the meat infusion which take part in the reaction would also greatly help in an understanding of its mechanism. Meat infusion contains autoxidizable substances which are able to reduce rH indicators by themselves (6); are these substances the ones which activate the system consisting of washed cells of Pneumococcus and glucose?

This is not a problem of theoretical interest only. The utilization of the nutrients in the process of growth is probably dependent upon what Quastel calls an "activation" of these nutrients by the cell under consideration (2). Is it not possible that the failure of Pneumococcus and other fastidious organisms to grow in synthetic media is due partly to the inability of these cells to "activate" the metabolites put at their disposal? It would be interesting to determine in what measure the ability to grow in synthetic media is related to the power of "activation" in the absence of meat infusion.

Experiment 8 indicates that glucose, in the presence of Pneumococcus cells, is able to reduce rapidly all the rH indicators, including indigo disulfonate. The reduction of glutathione by the Pneumococcus-glucose system is another evidence that this system can reach very negative potentials. Too little is known as yet to appreciate the significance of this reduction; however, its importance is suggested by the fact that thiol groups are essential for the growth of Pneumococcus[3] and that the addition of reduced cysteine to the medium always increases the rate of growth.

[3] Unpublished observations.

The formation of peroxide by glucose in the presence of Pneumococcus cells affords an explanation of Cole's (4) finding in 1914 that, although washed cells of Pneumococcus do not change hemoglobin to methemoglobin in the presence of air, they do so when traces of dextrose, or of certain other substances are added to the mixture. It is interesting that the substances which exhibit this property, also form peroxide in the presence of Pneumococcus cells. Ribose and inulin which fail in this particular are inactive in both instances. Since Avery and Neill (5) have shown that peroxide and methemoglobin formation are the expressions of the same mechanism, the relation between the presence of dextrose and the oxidation of hemoglobin appears evident.

Finally, it may be proper to point out briefly the significance of these observations for the characterization of bacterial cultures by their reducing properties. The bacteriological literature offers a great number of observations concerning the "reducing power" of different species of microorganisms. It is now known that sterile media themselves develop potentials corresponding to a great intensity of reduction (6, 9, 10); Coulter (11) has recently shown that typhoid bacilli, by using up the oxygen in solution in a medium, bring about the establishment of a potential similar to that which would develop after deaeration of the same sterile medium. The experiments reported here indicate that the amount and velocity of reduction of a dye in a culture depend largely on the presence of certain metabolites. What is, then, the "reducing power" of a tissue and of a culture? Is it only an expression of its ability to activate the molecules of the substrate, or does the protoplasm possess reducing properties of its own, determined by its physical and chemical constitution?

SUMMARY

The reducing power of plain broth cultures of Pneumococcus is largely dependent upon the presence in the medium at the time when the reduction test is performed of certain metabolites.

The washed cells of Pneumococcus are able to reduce the various indicators of oxidation-reduction potentials in the presence of glucose. The relative velocity of reduction of these indicators is determined by the number of cells used in the test, the concentration of the dyes, and their position in the oxidation reduction scale.

Oxidized thiol compounds (glutathione, cystine, oxidized thioglycollic acid) are likewise rapidly reduced by glucose in the presence of washed cells of Pneumococcus.

This Pneumococcus-glucose system is able to form peroxide under aerobic conditions. Those substances which form peroxide in the presence of Pneumococcus cells are also the ones which Cole found to be active in changing hemoglobin into methemoglobin under the same conditions.

The power of washed cells of Pneumococcus to reduce methylene blue in the presence of glucose is dependent on at least 2 constituents: one which can be readily removed from the cell by washing. Sugar-free meat infusion will function instead of it. The other is inactivated more slowly by the process of washing and is destroyed by 10 minutes heating at 55°C.

The interreaction between the glucose and the cell seems to result in a fundamental reaction in which one molecule of glucose becomes able to reduce rapidly one molecule of methylene blue. The existence of side-reactions often obscures this-ratio.

The significance of these observations is considered in relation to the nature and mechanism of the "activation" of metabolites, the preparation of synthetic media, the phenomena of growth, and the meaning of the expression "reducing power of a bacterial culture."

ADDENDUM

There has just appeared in the *Compt. Rend Acad. Science* an article by R. Wurmser and J. Geloso (12) in which these authors present data indicating that, under anaerobic conditions, the glucose molecule changes to a new form developing a reducing intensity corresponding to rH $= 8.2$ at pH 7. This form seems to give rise to a reversible system of oxidation-reduction, with an rH $\fallingdotseq 15$ corresponding to a mixture of 50 per cent oxidant-50 per cent reductant. Even in the presence of oxygen, the rH of this system does not go higher than 24.

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EXPERIMENTAL PNEUMONIA IN GUINEA PIGS

II. EFFECT OF ANTI-AUTOLYSATE SERA ON PNEUMOCOCCUS PNEUMONIA IN GUINEA PIGS

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In a previous communication (1) it was shown that extensive pneumonia associated with unrestrained multiplication of the organisms may be produced regularly in guinea pigs by the intratracheal injection of sublethal doses of certain toxic autolysates (pneumotoxin) mixed with sublethal doses of living pneumococci. Neither the pneumococci alone nor the toxic autolysate alone produced a comparable condition.

In the present paper, experiments are reported which demonstrate (a) that under certain conditions, anti-autolysate sera prepared in rabbits or horses by immunization with sterile filtrates of pneumotoxin prevent the development of pneumonia in animals inoculated with mixtures of pneumococci and autolysate; (b) that the protection against the development of pneumonia exhibited by these sera is heterologous, at least as regards Types I and II; (c) and that certain anti-pneumococcus horse sera used in the treatment of pneumonia in man contain either no heterologous pneumonia-preventing antibodies or slight amounts only.

EXPERIMENTAL

Methods

Immunization of Animals.—Rabbits were immunized over a period of 2 or 3 months by intracutaneous, subcutaneous or intravenous injections of sterile Berkefeld filtrates of the toxic autolysates from either Type I or Type II pneumococci, each rabbit being inoculated with the autolysate of one type only. The horses were immunized with subcutaneous injections of either mixed autolysate filtrates from Pneumococcus I, II and III or with mixed autolysates and the living organisms of all three types. The anti-autolysate sera from the horses were concentrated and refined by the usual methods and in this form tested for their

pneumonia-preventing properties.   Normal rabbit or normal horse sera were used as serum controls in the work.

The pneumococcus strains—Types I and II—were the same as those used in our previous work.   A strain of Pneumococcus III was also used.   This was isolated from a patient with pneumonia in the Presbyterian Hospital in May, 1928.   The method of preparing the toxic autolysates has been described in previous papers (1, 2).   Only those filtrates from Types I, II and III were used which would kill guinea pigs within 24 hours in doses of 0.2 cc. when injected intratracheally.

Young guinea pigs weighing from 190 to 250 gm. were used, and owing to the fact that the susceptibility to the autolysate usually varies inversely with the weight of the pig, it was found important to have the animal in each experiment as nearly the same weight as possible.

The injections of serum were given intraperitoneally 14 to 24 hours before the intratracheal infecting dose of pneumococci and autolysate.   At first, the rabbit immune sera were given intraperitoneally 24 hours previous to the intratracheal dose of organisms and pneumotoxin, but some of these sera caused the development of anaphylactoid reactions immediately after the intratracheal inoculations.   In later experiments the serum injections were made 14 to 18 hours before the intratracheal dose and in this way the anaphylactoid reactions were avoided.   Our anti-pneumotoxic horse sera have never given anaphylactic sensitization, even when the sera were given 24 hours before the infecting dose of toxin and pneumococcus (3).

Intratracheal Injections.—In carrying out the pneumonia preventing tests, the proper adjustment of the doses of pneumotoxin and pneumococcus is essential. The dose of pneumococcus used was about one half that which would cause death from septicemia when injected intratracheally without mixture with autolysate. The same principle governs the dose of autolysate used in these experiments.   The amount employed was one half the quantity necessary to kill a pig of approximately the same weight in 24 hours.   With experience as to the virulence of the culture and the strength of the autolysate, it is comparatively easy to obtain the proper amounts of autolysate and pneumococci for these experiments.

The intratracheal injections were carried out as follows:—An 18–24 hour broth culture of the pneumococcus was diluted with broth to the strength desired.   This tube, containing the diluted pneumococcus culture, was kept in ice water throughout the experiment.

A tube containing the toxic filtrate was chilled, the vaseline seal opened, and the filtrate pipetted into a narrow, chilled tube.   This tube, containing the toxic autolysate, was also kept in ice water throughout the experiment.   Just before the injection, 0.2 cc. of the diluted pneumococcus culture and 0.2 cc. of the autolysate were pipetted into another iced tube, mixed, and 0.2 cc. of the mixture was drawn into an iced syringe and immediately injected intratracheally into a guinea pig.   A fresh mixture was always prepared for each injection and the same precautions as to chilling, etc., were carried out with both control preparations consisting of pneumococcus and broth, and autolysate and broth.

Protective Action of Anti-autolysate Sera Against the Pneumonia Caused by Pneumococci Plus Toxic Filtrates of Homologous Type

Table I demonstrates the protective action of two preparations of concentrated anti-autolysate horse sera against pneumonia caused by mixtures of toxic autolysate Type I and living pneumococcus Type I.

This experiment shows that two pigs injected intraperitoneally with 1 cc. each of normal horse serum 18 hours previous to the inoculation of

TABLE I

Action of Anti-autolysate Sera in the Prevention of the Pneumonia Caused by Pneumococci and Toxic Filtrates of Homologous Type

Pneumococcus culture Type I; very slight growth. Autolysate from Type I. 0.2 cc. pneumococcus, Type I culture + 0.2 cc. autolysate. 0.2 cc. inoculated intratracheally; Concentrated autolysate horse 30336. 9/24/28. Concentrated autolysate horse 30337. Normal horse serum.

No.	Wt.	Intraperitoneal injection 10/1	Intratracheal injection 10/2	Symp-toms	Died or survived	Cultures		Extent of lung consoli-dation
						Heart	Lung	
55	238	1 cc. normal horse serum	Pn. + autolysate	+++	D. 3 days	+++	+++	++
58	230	1 cc. 30336	Pn. + autolysate	0	S.	—	—	—
69	232	1 cc. 30337	Pn. + autolysate	0	S.	—	—	—
61	226	—	Broth + autolysate	+	S.	—	—	—
64	224	—	Pn. + broth	0	S.	—	—	—
54	230	1 cc. normal horse serum	Pn. + autolysate	+++	D. 2 days	+++	+++	+++
56	232	1 cc. 30336	Pn. + autolysate	0	S.	—	—	—
65	238	1 cc. 30337	Pn. + autolysate	0	S.	—	—	—
57	222	—	Broth + autolysate	+++	S.	—	—	—
60	222	—	Pn. + broth	0	S.	—	—	—

pneumococci and toxic autolysate died in 2 and 3 days respectively, with pneumonia and septicemia; while four pigs previously prepared with 1 cc. each of two preparations of concentrated anti-autolysate sera remained well. The difference in the appearance of the protected and unprotected animals was striking. 18 hours after the intratracheal injection of pneumococcus and autolysate the two pigs which had received the normal serum were severely dyspnoeic and appeared extremely sick, while the pigs which had received the anti-autolysate

serum had normal respiration and appeared well.   The two pigs which were injected with the autolysate and broth were dyspnoeic, one being desperately sick for 8 days but finally recovering; while the two which were injected with the pneumococcus broth culture remained well.

Serum preparation 30336 was obtained from two horses immunized with toxic autolysate filtrates and contained no specific protective substances.   Serum preparation 30337 was obtained from two horses immunized with toxic autolysates and living pneumococci and protected Pn. 1,10^{-4}.   The question of the importance of pneumococcus specific protective properties in these experiments will be considered later.

Protective Action of Anti-autolysate Sera on the Pneumonia Caused by Pneumococci Plus Toxic Filtrates of a Heterologous Type

That the pneumonia preventing action of our anti-autolysate sera is heterologous as regards pneumococcus Types I and II has been demonstrated conclusively many times.   These experiments were carried out with anti-autolysate sera produced in rabbits by the immunization with autolysates of one type only (Type I or Type II).   The experimental pneumonia in each instance was caused by the autolysate and pneumococci of heterologous strains (Type I or II).   Table II demonstrates the preventive action of Type I anti-autolysate serum against pneumonia caused by pneumococcus and autolysate Type II, and is self explanatory.   The same pneumonia-preventing action has been demonstrated for rabbit anti-autolysate Type II sera against the pneumonia caused by injections of Pneumococcus I toxin and living pneumococcus I.

These experiments therefore demonstrate that our anti-pneumotoxic sera are definitely protective against pneumonias caused by mixtures of pneumococci and autolysates of a heterologous strain.

Action of the Anti-pneumococcus Sera Used in the Treatment of Human Pneumonia for the Pneumonia-preventing Heterologous Substances

In testing the anti-bacterial pneumococcus sera for the presence of the pneumonia-preventing substances which have been shown to be present in our anti-autolysate sera, it was important to rule out the

effect of the specific protective substances which all these anti-bacterial sera contain. To accomplish this, it was only necessary to test out the efficacy of these sera against the pneumonia produced by a pneumococcus and autolysate from a heterologous strain.

Table III gives one of our experiments of this kind. It is seen that the serum concentrated by the Felton method shows no heterologous pneumonia-protective powers; the serum produced by the Zinsser

TABLE II

Action of an Anti-autolysate Serum in Preventing Pneumonia Caused by Pneumococci Plus Toxic Filtrates of a Heterologous Type

Pneumococcus II—good growth. Diluted 1–10 with broth Autolysate II. 0.3 cc. Pn. II dilution + 0.1 cc. autolysate. 0.2 cc. mixture injected. Controls: (a) 0.3 cc. Pn. + 0.1 cc. broth—0.2 cc. injected. (b) 0.3 cc. broth + 0.1 cc. autolysate—0.2 cc. injected. Rabbit serum 7–54, Anti-autolysate I.

No.	Wt.	Intraperitoneal injection 5/9	Intratracheal injection 5/10	Symptoms	Died or survived	Cultures		Extent of lung consolidation
						Heart	Lung	
10-11	204	2 cc. normal rab.	Pneumococcus + autolysate	+++	D. 40 hrs.	+++	+++	+++
8-74	202	2 cc. 7–54	Pneumococcus + autolysate	0	S.	—	—	—
8-88	204	2 cc. 7–54	Pneumococcus + autolysate	0	S.	—	—	—
8-89	210	—	Pneumococcus + autolysate	+++	D. 40 hrs.	+++	+++	+++
10-09	206	—	Pneumococcus + autolysate	+++	D. 18 hrs.	+	+++	+++
8-87	200	—	Broth + autolysate	0	S.	—	—	—
10-06	206	—	Pneumococcus and broth	0	S.	—	—	—

method shows a slight amount (protected one of the two pigs injected), while the rabbit anti-autolysate serum gives perfect protection. The same results were obtained in another experiment carried out in a similar way. Still another anti-pneumococcus serum used in treating human pneumonia was tested for heterologous pneumonia-preventing substances, and proved to be even weaker in these properties than the serum produced by the Zinsser method.

These experiments demonstrate how poor in heterologous anti-pneumonia antibodies the ordinary anti-bacterial pneumococcus sera are.   The explanation for the complete absence of these antibodies in the serum produced by the Felton method is probably to be found in its method of preparation, which concentrates the specific protective

TABLE III

Action of Certain Anti-pneumococcus Sera Used in the Treatment of Human Pneumonia in Preventing the Pneumonia Caused by Autolysate and Pneumococci of a Heterologous Type

Pneumococcus Type II; slight growth on broth.   Autolysate Type II.   0.2 cc. Pn. culture + 0.2 cc. autolysate.   0.2 cc. injected.   Controls: Pn. + broth and autolysate and broth.   Sera: Felton's antibodies I; Zinsser anti-pneumococcus serum; Rabbit 7–54; Anti-autolysate I.

No.	Wt.	Intraperitoneal injection 3/2	Intratracheal injection 3/3	Symptoms	Died or survived	Cultures		Extent of lung consolidation
						Heart	Lung	
7-59	200	2 cc. Felton antibodies	Pn. + autolysate	+++	D. 2 hrs.	0	+	++
7-77	200	Same	Pn. + autolysate	+++	D. 2 days	++	+++	+++
7-67	200	2 cc. Zinsser serum	Pn. + autolysate	0	S.	−	−	−
7-78	205	Same	Pn. + autolysate	+++	D. 40 hrs.	++	+++	+++
7-73	200	2 cc. 7–54	Pn. + autolysate	++	S.	−	−	−
7-66	200	2 cc. 7–54	Pn. + autolysate	+	S.	−	−	−
7-69	195	2 cc. normal horse	Pn. + autolysate	+++	D. 18 hrs.	+++	+++	+++
7-75	200	2 cc. normal rabbit	Pn. + autolysate	+++	D. 5 hrs.	0	+	++
7-71	190	—	Pn. + autolysate	+++	D. 40 hrs.	+++	+++	+++
7-80	195	—	Broth + autolysate	+	S.	−	−	−
7-74	190	—	Pn. + broth	0	S.	−	−	−

bodies in the globulin fraction of the serum and discards that part of the serum which contains neutralizing antibodies (the pseudoglobulin fraction.)

It is logical to suppose that a serum containing specific protective antibodies, would have a preventive effect by its antibacterial properties, on the pneumonia caused by the homologous type of organism and autolysate; and that this preventive effect would correpond closely to

the amounts of specific protective substances which these sera contain. This was found to be the case. The serum produced by the Felton method, containing 1000 protective units, protected 75 per cent of the animals injected; the Zinsser serum protected 50 per cent, and the other Anti-pneumococcus I serum only delayed death in one instance. To save space, a table giving the details of these experiments is omitted.

Protective Effect of Normal Rabbit or Horse Serum

Occasionally normal rabbit serum protects against the pneumonia produced by a mixture of living pneumococcus and autolysate. We believe this to be due to a non-specific reaction induced by the foreign serum in a small percentage of the injected pigs (about 8 per cent). Horse serum occasionally shows this non-specific protective action. Stillman had the same experience in his pneumococcus work in mice. He found that normal rabbit serum injected intraperitoneally in mice sometimes protected them from pneumococcus infections. These rabbit sera contained no pneumococcus specific protective substances (4).

The Content of Pneumococcus Specific Protective Substances in the Anti-autolysate Sera

That the pneumonia-preventing properties of anti-autolysate sera are not related to the pneumococcus specific protective substances was demonstrated conclusively by the fact that the anti-autolysate sera are equally effective against a pneumonia due to the toxin and pneumococci of either a heterologous or homologous strain. We also titrated all our rabbit anti-autolysate sera and in no instance did they show the presence of specific protective substances for mice. Neither were there specific protective substances in horses immunized with the autolysate filtrates, but protective substances for pneumococci Types I and II were present in small amounts in the sera obtained from the horses which had been injected with living pneumococcus Types I, II and III, in addition to the autolysates.

DISCUSSION

The experiments reported in this paper appear to indicate that under certain conditions the sera produced in rabbits or horses by immuniza-

tion with Berkefeld filtrates of certain anaerobically produced autolysates of pneumococcus Type I or II protect against the pneumonia caused in guinea pigs by the intratracheal injection of mixtures of living pneumococci and toxic autolysates.   Since the sera contain no specific anti-bacterial antibodies, it is evident that their protective action must be due to some other form of immune body.   The immune body present differs also from the specific anti-bacterial bodies in that it is apparently identical for both Types I and II of the pneumococci and is therefore not type specific.

Whether this lung-toxic substance is produced in spontaneous pneumonia, or in the experimental pneumonia produced by intratracheal injections of pneumococci alone—as in the dog or monkey—is a question which cannot be answered at present.   The demonstration that a toxic substance of this nature is present in these conditions would help to explain some of the obscure features of pneumococcus pneumonia in animals and man.

In the production of curative sera for lobar pneumonia, most of the efforts have been devoted to obtaining sera with a high content of specific protective antibodies against living organisms, but Cecil and Blake (5) have found that the serum of a monkey may be completely free of any specific protective substance and yet be highly immune to pneumonia.   The reverse was also found to occur.   A monkey whose serum may protect mice against 100 to 1000 M.L.D.'s of pneumococci may still be susceptible to the homologous pneumococcus pneumonia.

In connection with this work, it is also of interest to note that Cecil and Blake demonstrated that resistance to experimental pneumonia in monkeys is to some degree species specific as regards pneumococci Types I, II and III (6).   Stillman (7) has come to the same conclusion in his work on the production of pneumococcus pneumonia in mice by the inhalation method.   He found that mice which had been rendered immune to one type of pneumococcus (Type I) by the inhalation method, may show an increased resistance locally when exposed to infection by the same method with organisms of another type (Type II).

<div align="center">CONCLUSIONS</div>
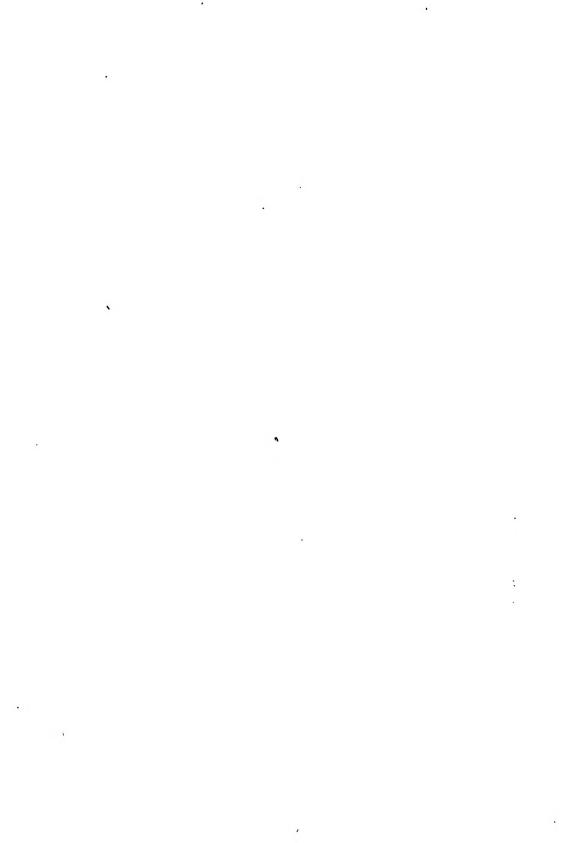
1. Anti-pneumotoxic sera prepared in rabbits or horses by immunization with sterile filtrates of the pneumotoxin, under certain condi-

tions protect against the pneumonia caused by the intratracheal injections of mixtures of living pneumococci and toxic autolysates.

2. The protection against the development of pneumonia is heterologous, at least as regards Type I, Type II, *viz.:* an anti-autolysate serum prepared by the immunization with a pneumotoxin from one type of pneumococcus will prevent the development of pneumonia caused by the injection of pneumococci and autolysate from another type.

3. Certain anti-pneumococcus horse sera used in the treatment of pneumonia in man, either contain no heterologous pneumonia-preventing antibodies or slight amounts only. These sera, however, protect against the pneumonia produced by injections of pneumococci and pneumotoxin of the homologous strain, the degree of protection depending on the amount of specific protective substances such sera contain.

4. Anti-pneumotoxic sera produced in rabbits or horses by the injection of sterile Berkefeld filtrates of the toxic autolysates contain no pneumococcus specific protective substances.

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STUDIES ON INFLAMMATION

I. Fixation of Vital Dyes in Inflamed Areas

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Inflammation involves local physiological changes in the permeability of capillaries, in the rate of blood flow, and perhaps in the normal balance of body fluids. Increase of capillary permeability is shown by local edema. With the passage of proteins from the plasma into the inflamed area the osmotic relationship between tissue fluids and blood plasma is doubtless disturbed. About 30 years ago Adler and Meltzer (1) stated that the passage of fluids from tissue spaces into lymphatics probably depends on osmosis perhaps assisted to some extent by filtration. Krogh (2) recently pointed out that the passage of proteins from capillaries increases the osmotic pressure of the tissue fluids. This change in an inflamed area would probably modify the passage of fluid into the regional lymphatics which drain the inflamed area.

The dissemination of foreign substances from the site of injection into the lymphatics and blood stream has been studied by various investigators. Muscatello (3) showed that carmine and various other inert particles when injected into the peritoneal cavity reach the anterior mediastinal lymph nodes very rapidly. Noetzel (4) injected bacteria (*B. pyocyaneus*) into the knee joints of rabbits. 5 to 10 minutes later he was able to demonstrate the presence of the organisms in the inguinal, crural, and lumbar lymph glands. Buxton (5) found that typhoid bacilli when injected into the peritoneal cavity, appeared in great numbers within a few minutes in the blood stream. Wells and Johnstone (6) showed that the absorption of bacteria from the peritoneal cavity takes place through lymphatic vessels.

Within recent years evidence has accumulated to show that an inflammatory reaction at the site of injection might be a factor in delaying the passage of foreign substances into the blood stream. Pawlowsky (7) demonstrated that the dissemination into the blood stream of staphylococci injected into a previously inflamed knee joint was either inhibited or wholly prevented. The studies of Issayeff had showed that peritonitis caused by a sterile irritant increased temporarily the resistance of the animal to subsequent intraperitoneal inoculation of bacteria (8). Opie (9, 10) showed that foreign protein injected into the dermis of an immunized animal was fixed at the site of injection; the contact of antigen and

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antibody at the site of injection causes an acute inflammatory reaction (Arthus phenomenon). In normal animals, on the contrary, foreign proteins when injected into the dermis caused little if any inflammatory reaction and readily penetrated into the blood stream. Willis (11) studied the dissemination of tubercle bacilli from the site of cutaneous inoculation and showed that in reinfected guinea pigs the spread of tubercle bacilli from the site of inoculation is retarded, whereas in normal animals the organisms pass readily to the regional lymph nodes. Opie (12) recently showed that acute inflammation of the peritoneal cavity caused by aleuronat retards the rush of injected hemolytic streptococci from the peritoneal cavity into the circulating blood and after 24 hours completely prevents it.

The present study was undertaken in order to determine whether various foreign substances passed to tributary lymphatic vessels when injected into an inflamed area. At first, substances consisting of large particles were used. Nucleated cells from fowl's blood were unsuitable for the experiment because they failed to pass into the regional lymphatics when injected into normal subcutaneous tissue. *Bacillus subtilis* was tried with the same negative results. It was in the course of these preliminary observations that trypan blue was tried. This vital dye when injected into the subcutaneous tissue of the extensor surface of the fore-leg of a rabbit appeared within 30 to 40 minutes in the lymphatic vessels of the axilla (13). The tributary afferent lymphatic vessels, the lymph node, and the efferent lymphatic vessel revealed plainly the presence of the dye. If, however, the dye was injected into an area in which an inflammatory reaction had been produced some time before, the dye did not appear in the tributary lymphatics. The afferent lymphatics, the lymph node, and the efferent lymphatic vessel remained colorless. The dye was evidently retained in the inflamed area.

The Retention of Trypan Blue in Areas of Inflammation

Sterile inflammation was produced by a mixture of 5 per cent of aleuronat and 3 per cent of starch in 0.5 per cent saline solution. Usually 5 cc. of this solution were injected into the subcutaneous tissue of the extensor surface of the fore-leg of a rabbit 2 or 3 cm. from the shoulder joint. Either immediately following the injection of aleuronat or after varying intervals of time, 3 cc. of a 1 per cent solution of trypan blue in saline were injected subcutaneously into the inflamed area. The same amount of dye was injected into the other fore-leg to serve as a control. The lymph from the efferent lymphatic vessel was collected as recently described by Freund and Whitney (14). To expose the lymphatic the pectoral muscles were

incised and the subclavian vein exposed, under ether anesthesia. Alongside and somewhat below the vein an efferent lymphatic vessel was seen to emerge from one of the axillary lymph nodes. The lymph was drawn by puncturing the efferent lymphatic and introducing a capillary pipette into it. On the normal side the lymph thus obtained from the efferent lymphatic was stained with the dye. Varying concentration of trypan blue in the lymph was more conspicuous when the capillary

TABLE I

Retention of Trypan Blue at the Site of Inflammation

Rabbit No.	Interval between injection of irritant and that of dye	Duration of inflammation	Presence of dye on normal side		Presence of dye on inflamed side	
			Lymph of efferent lymphatic	Lymph node	Lymph of efferent lymphatic	Lymph node
	hrs. : mins.	*hrs. : mins.*				
1	0:00	1:30	++	++	trace	trace
2	0:00	2:00	+	+	+	+
3	0:00	2:20		trace		faint trace
4	0:30	3:30	+	+	0	0
5	1:00	3:40	++	++	0	0
6	2:10	4:30	+	+	0	0
7	4:00	6:00	+	+	0	0
8	19:00	20:30	++	++	0	0
9	19:30	20:30	++	++	0	0
10	20:30	23:00	+	+	faint trace	0
11	22:00	23:00	++	++	0	faint trace
12	22:30	23:00	+		0	0
13	22:30	23:30	+	+	0	0
14	23:30	28:30	+	+	0	0
15	24:00	25:30	+	+	faint trace	trace
16	25:00	26:00	++	++	faint trace	trace
17	25:00	26:00	faint trace		0	
18	26:00	27:00	++	++	trace	+
19	46:00	47:30	+++	+++	0	0
20	46:15	48:00	+	++	trace	0
21	46:15	48:15	+	++	0	faint trace

pipettes containing lymph were held against a white background. The axillary lymph nodes were exposed and removed in order to determine if they were stained by the dye.

The results of this series of experiments appear in Table I. A colorless lymph is indicated by zero; a distinctly blue color by one plus (+); a dark blue color by two plus signs (++); and a bluish-

black color by three plus signs (+ + +). "Trace" or "faint trace" of blue refers to barely perceptible staining of lymph or lymph nodes.

When trypan blue was injected in both fore-legs of the animal immediately after the inflammatory irritant, the passage of the dye occurred in both the normal and inflamed side with about equal intensity, but in Rabbit 1 the concentration of the dye was less on the inflamed side. If, however, the dye was injected only 30 minutes after the irritant (Rabbit 4), trypan blue did not pass either into the lymph node or its efferent lymphatic vessel on the inflamed side. Evidently the fixation or retention of the dye in an inflamed area occurs very early and is seen at least 30 minutes after the onset of inflammation. The fixation of the dye at the site of inflammation was proven not merely by the absence of pigment in the tributary lymph node but by its failure to appear in the lymph from the afferent lymphatic vessels on the inflamed side and its presence on the normal side in four rabbits. Lymph was in most instances obtained 1 to 2 hours after the injection of the dye. In one animal (Rabbit 14) in which the lymph was collected after 5 hours the result was the same, i.e., fixation of the dye in the inflamed area.

The Accumulation of Trypan Blue in Inflamed Areas

Since trypan blue tends to remain in situ when injected directly into the inflamed area, the attempt was made, at the suggestion of Dr. Eugene L. Opie, to determine whether the dye injected into the blood stream would accumulate at the site of inflammation.

A few years ago Lewis (15) found that if the cornea of a rabbit is inoculated with a living culture of the tubercle bacillus, a progressive lesion results characterized by an intense congestion of the conjunctiva. 24 hours or more after such an inoculation, if the animal is injected intravenously with trypan red, the fluid in the anterior chamber of the inoculated eye always becomes colored. He also found that an intense conjunctivitis occurs when abrin is dropped in the eye. Here again, when trypan red or trypan blue is injected intravenously at different stages of this inflammation, the dye appears rapidly in the anterior chamber and in the edematous conjunctiva. Ramsdell (16) injected trypan blue into the veins of rabbits and guinea pigs previously treated with foreign serum and found that injection of the same serum into the skin of the ear, immediately caused local infiltration of the dye into the adjacent tissue. She regarded this infiltration of

the dye as an indicator of edematous changes resulting from toxic injury to the capillary endothelium.

Okuneff (17) found that the application of a thermal irritant favors the passage of vital stains from the blood stream into the area heated. Kusnetzowsky (18) also observed that the local application to the skin of an irritant such as heat or mustard oil causes an accumulation of trypan blue in the inflamed area when the dye has been previously injected into the blood stream. He found the dye to be concentrated both in the cells of the connective tissue and especially in the histiocytes.

An inflammation was produced by injection of aleuronat into the subcutaneous tissue of the fore-leg of a rabbit. A 1 per cent solution of trypan blue was injected intravenously in varying quantities ranging from 5 to 10 cc. In three rabbits the dye was injected intravenously immediately after injection of the inflammatory irritant into the fore-leg. In the remaining experiments the dye was injected at various intervals of time after the inflammatory irritant. When the inflammatory reaction had proceeded for varying periods of time, as shown in Table II, the area of inflammation and the tributary lymph node were exposed and compared with the corresponding normal areas on the opposite fore-leg to determine the presence of trypan blue. Even when the dye was injected intravenously immediately after the local injection of irritant, there was a greater accumulation of trypan blue in the inflamed area than in the corresponding normal area. On the inflamed side, not merely the deep fascia was more intensely stained by the dye but the muscle tissue as well. The time element in the appearance of the dye in the lymph node on the inflamed side was noteworthy. When the dye was injected immediately after the irritant, the dye appeared in the lymph node in large amounts; but if the dye was injected only $1\frac{1}{2}$ hours after the inflammatory irritant (Rabbit 25, Table II), less of it seemed to reach the lymph node of the inflamed than that of the normal side. The longer the interval of time between the injection of the irritant and that of the dye, the more efficient appeared to be the retention of trypan blue by the inflamed area, and consequently less of the dye diffused to the tributary lymph node.

A series of experiments was performed in which the inflammatory irritant used was broth (concentrated to 1/14 of its original volume) and injected into the dermis of the abdomen. The dosages ranged

from 0.1 cc. to 0.5 cc. After varying intervals of time, 5 to 10 cc. of 1 per cent trypan blue were injected intravenously. In some rabbits the dye was injected immediately after the intracutaneous injection of broth. Within usually less than 1 hour after introduction of the dye the small inflamed areas of the dermis appeared intensely blue and generally stood out prominently on the surface of the abdomen. A few experiments were repeated with bacteria acting as the inflamma-

TABLE II

Accumulation of Trypan Blue in Inflammatory Areas

Rabbit No.	Interval of time between injection of irritant and that of dye	Duration of inflammation	Presence of dye on normal side		Presence of dye on inflamed side	
			Normal area corresponding to area of inflammation	Lymph node	Inflamed area	Lymph node
	hrs.:mins.	*hrs.:mins.*				
22	0:00	1:15	trace	+	++	trace
23	0:00	3:20	trace	++	+++	++
24	0:00	19:00	+	trace	+++	++
25	1:30	24:00	trace	+++	++	+
26	2:30	28:00	trace	++	++	+
27	4:00	6:30	faint trace	0	+++	0
28	4:30	6:30	trace	trace	+++	faint trace
29	4:30	24:00	+		+++	+
30	5:45	7:00	0	faint trace	+++	faint trace
31	19:00	20:00	trace	++	+++	faint trace
32	20:45	21:30	+	faint trace	+++	trace
33	21:05	22:00	faint trace	0	+++	0
34	24:00	47:00	0		+	0
35	45:45	47:30	faint trace	++	++	faint trace
36	45:50	47:00	faint trace	trace	++	trace

tory irritant. 0.1 cc. of a saline suspension of *Staphylococcus aureus* was injected into the dermis of the abdomen followed a few hours later by intravenous injection of trypan blue. Within a relatively short time the tissues in the site of inflammation appeared markedly stained. In many cases these areas showed a pale reddish central zone surrounded by an intensely dark blue band measuring about 0.5 cm. in width. The localization of intravenously injected trypan blue in inflamed areas of the dermis was evident in 32 out of 36 experiments.

Rabbit	Duration of inflammation	No. of inflamed areas on abdomen	No. of experiments in which inflamed areas were stained blue by the fixation of trypan blue
	hrs.:mins.		
33	0:38	4	4
31	0:45	2	2
22	1:15	4	4
37	1:00 to 3:30	6	6
26	1:30 to 4:30	5	3
25	3:00	3	3
38	4:00	4	4
34 (aleuronat)	6:30	5	4
39	21:05	3	2

The central zone which has a red congested appearance and which is often seen in these intracutaneous areas of inflammation is perhaps due to thrombosis of small vessels. Histological sections of such areas reveal some thrombosed vessels with acute inflammatory changes in the surrounding tissue.

The Fixation of Trypan Blue by an Inflammatory Reaction in the Peritoneal Cavity

It is well known that the retrosternal lymph nodes of the anterior mediastinum drain the peritoneal cavity. Years ago Muscatello (3) pointed out that carmine and various inert particles injected intraperitoneally into dogs reach the anterior mediastinal lymph nodes very rapidly. Okuneff (19) recently pointed out that certain substances injected intraperitoneally such as animal charcoal, casein or gelatine delay the absorption into the blood stream of trypan blue when the dye is injected in the abdominal cavity either simultaneously or within thirty minutes after the injection of these substances. The mechanism involved was thought to be an adsorption of the dye by these substances. As already mentioned Opie (12) has recently reported experiments in which hemolytic streptococci, injected into the peritoneal cavity of rabbits, appeared within 10 minutes in the blood stream. If 24 hours prior to the injection of these bacteria a sterile inflammatory irritant, such as aleuronat, had been injected into

the peritoneal cavity, the organisms were prevented from reaching the blood stream.   In view of this interesting study, trypan blue was injected into the peritoneal cavity of normal rabbits and into that of animals which had previously received an intraperitoneal injection of aleuronat.

About a half hour after the injection of the dye into the peritoneal cavity of normal rabbits, the pigment was found in the lymph of the retrosternal lymphatics.   The retrosternal lymph nodes also appeared

TABLE III

Retention of Trypan Blue by Inflammation in the Peritoneal Cavity

Experiment	Interval of time between injection of irritant and that of dye	Duration of inflammation	Presence of dye in the retrosternal lymph nodes	
			Injected trypan blue controls	Animals with peritoneal inflammation
	hrs.:mins.	*hrs.:mins.*		
1	0:00	0:34	++	++
2	2:00	2:33	++	+
3	5:08	6:00	++	0
4	22:45	28:00	+++	trace
5 (Neutral red)	23:00	24:00	++	0
6	23:10	49:00	+++	++
7	24:00	25:40	+++	trace to +
8	24:00	29:00	+++	trace
9	27:30	29:20	+++	faint trace
10	27:55	30:00	+++	+

deeply stained with the dye.   The peritoneal cavity was stained only moderately.   However, when trypan blue was injected into the inflamed peritoneal cavity, the retrosternal lymphatics and the retrosternal lymph nodes either failed to show the presence of the dye or occasionally showed it only in traces.   The peritoneal cavity was much more deeply stained than in normal animals.   Clumps or precipitates of the dye were conspicuous on the omentum and on the peritoneal surface of the diaphragm.

The amount of aleuronat used was either 5 cc. or 10 cc.   The dye was injected immediately after the irritant or else after varying intervals of time.   Usually 5 cc. of 1 per cent trypan blue were injected

intraperitoneally. After varying periods of time the retrosternal lymph nodes of normal and injected animals were examined for the presence of the dye. The results are shown in Table III. When the dye was injected immediately after the irritant, the retrosternal lymph nodes of normal animals and of those with an inflamed peritoneal cavity were equally colored by the dye (Experiment 1). If, however, the inflammation had been in progress as long as 2 hours when the dye was injected, the appearance of trypan blue in the retrosternal lymph nodes was conspicuously less than in the lymph nodes of the control animal. The dye is evidently prevented from leaving the peritoneal cavity by the inflammatory reaction. This fixation of dye is analogous to that of hemolytic streptococci observed by Opie (12).

CONCLUSIONS

Trypan blue injected into normal subcutaneous tissue passes rapidly to the regional lymphatic node and is found in lymph drawn from its efferent lymphatic.

When the dye is injected into the normal peritoneal cavity it rapidly appears in the lymph of the retrosternal lymphatics and stains deeply the retrosternal lymphatic nodes.

Trypan blue injected into the site of inflammation in the subcutaneous tissue or in the peritoneal cavity is fixed in the inflamed area and fails to reach the regional lymphatic nodes.

If an inflammatory reaction has been produced in the dermis or in the subcutaneous tissue, trypan blue injected into the circulating blood enters the site of inflammation and is fixed so that the tissues are deeply stained.

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OBSERVATIONS CONCERNING THE PERSISTENCE OF LIVING CELLS IN MAITLAND'S MEDIUM FOR THE CULTIVATION OF VACCINE VIRUS

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PLATE 7

The fact that vaccine virus is capable of multiplying in cultures of susceptible tissues is well established. Some investigators (1), however, have claimed that it is possible to obtain an increase of this infectious agent in the absence of living host cells. These claims have not been substantiated. Indeed, Harde (Steinhardt) (2, 3) and Nye and Parker (4) have reported that tissues killed by freezing and thawing and by hypotonic salt solutions did not support the survival or multiplication of vaccine virus.

Recently Maitland and Maitland (5) recorded observations concerning "cultivation of vaccinia virus without tissue culture." Their medium consisted of minced chicken kidney suspended in a mixture of chicken serum (1 part) and Tyrode's solution (2 parts). These workers found that the virus increased in the absence of any detectable growth of cells in the cultures. In fact, they state that "after 24 hours the small pieces of kidney had begun to disintegrate, and by the third day autolysis of the tissue was extensive." There is no reason to doubt that vaccine virus multiplied in Maitland's medium. Moreover, Eagles and McClean (6) and Andrewes (7) have shown that certain viruses are capable of increasing under such conditions. Inasmuch as most workers have been unable to cultivate vaccine virus in the absence of living cells, and since it is known that certain cells remain viable under many conditions (8, 9), there are reasons for ascertaining whether living cells can persist or whether growth of cells can occur in Maitland's medium. It is with this problem that the present communication is chiefly concerned.

EXPERIMENTAL

Methods and Materials

Vaccine Virus.—Levaditi's neurovaccine was injected in the testicles of a rabbit. 4 days later the testicles were removed[1] and ground in a mortar with sand and M/50 phosphate solution, pH 7.6. The emulsion was thoroughly centrifuged and the supernatant fluid was used as an inoculum. The titer of the virus was determined in rabbits by means of intradermal inoculations of 0.2 cc. of serial dilutions of virus emulsions.

Culture Medium.—Throughout this work Maitland's technique of preparing and testing cultures was followed.

"Blood was withdrawn from a hen and after it had clotted the serum was collected The hen was killed with chloroform and ether, and the kidneys, which were removed aseptically, were minced finely with scissors. Into a flask was put 0.66 c. cm. (approximately) of minced kidney and 1.33 c. cm. of inoculum diluted 1 in 6.6 with Tyrode's solution. The mixture was allowed to stand in the cold room for four hours. Then were added 12 c. cm. of Tyrode's solution and 6 c. cm. of hen's serum. The final dilution of the inoculum was thus 1 in 100. The mixture was distributed in 2 c. cm. into Carrel's tissue culture flasks, type D, which were incubated aerobically at 37°C., without caps. The cultures were tested after various periods of incubation by grinding the whole of the contents of each flask with sand and centrifugalising."

Controls.—In addition to the normal kidney tissue, controls with minced kidney tissue that had been frozen (CO_2 snow) and thawed 10 times were employed. To determine whether the tissues were living or dead at the beginning of each experiment, just prior to the distribution of the cultures in flasks, bits of the normal and of the frozen kidney tissue were placed in hen plasma and embryo extract on mica coverslips which were inverted and sealed over hollow ground slides. These preparations were then incubated at 37°C. After 5 or 6 days they were examined for evidences of cell growth.

To ascertain whether living cells persisted in the cultures prepared and handled according to Maitland's method, bits of tissue were removed from the Carrel flasks at 3, 4, and 5-day intervals, washed in Ringer's solution, and planted in plasma and embryo extract on mica coverslips These preparations were examined frequently for evidences of cell growth.

Inasmuch as it seemed possible that certain cells might at times multiply in a mixture of serum and Tyrode's solution, bits of normal kidney tissue were placed in such a mixture on mica coverslips over which hollow ground slides were placed and sealed. The slides were not inverted, since it is well known that in a liquid medium cells usually require a surface along which to grow. After incubation at 37°C., these preparations were examined for evidences of cell migration or cell growth.

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[1] All operations were performed under ether anesthesia.

To test the viability of bacteria one usually resorts to subcultures rather than to examinations of fresh and stained specimens. Consequently, in determining whether cells are able to remain alive in the medium employed by Maitland for the cultivation of vaccine virus, subcultures, as described above, of bits of tissue in a favorable medium were made. Under these conditions, it is appreciated that positive results are more significant than negative ones. In this work 4 experiments were performed, 3 of which will be described in detail. The fourth will be omitted, since the results were similar to those of the others.

Experiment I

Jan. 15, 1929.—Fresh hen kidney was minced. One portion was frozen (CO_2 snow) and thawed 10 times. Then both portions were placed in contact with vaccine virus in the ice box for 4 hours. After the exposure to virus, 10 pieces of unfrozen and frozen tissue respectively were cultured as controls in plasma and embryo extract on mica coverslips. The inoculated frozen and unfrozen tissues were then added to a mixture of serum and Tyrode's solution and distributed in Carrel flasks (2 cc. each)—3 with unfrozen and frozen tissue respectively. Titer of vaccine virus in the cultures at this time: frozen = 1:100; normal or unfrozen = 1:100.

Jan. 17.—Each of the 10 control cultures made on coverslips from the unfrozen bits of tissue showed growth of cells, while in those made from frozen tissue no evidence of cell growth was observed at this time or upon subsequent examinations.

Jan. 18.—The cultures were removed from the Carrel flasks. Bits of tissue from 2 of the flasks with unfrozen kidney and from 2 of the containers with frozen kidney were washed in Ringer's solution and subcultured in hen plasma and embryo extract. None of the subcultures of frozen tissue showed growth in 6 days (Fig. 2), while in all of those made from unfrozen tissue growth of cells (Fig. 1) was evident. The predominant cells were fibroblasts, yet cells of the macrophage type evidencing phagocytosis were observed.

The titer of vaccine virus in the flasks with frozen (F) and unfrozen (N) tissues was as follows: N1 = 1:500, N2 = 0, N3 = 1:5,000; F1 = 0, F2 = 0, F3 = 1:50.

The results of the above experiment indicate that vaccine virus did not multiply in the presence of kidney tissue killed by freezing and thawing, while it did persist or increase in amount in cultures set up with normal kidney tissue in a mixture of serum and Tyrode's solution. Furthermore, it is quite obvious that living cells persisted for at least 3 days in Maitland's medium.

Experiment II

Feb. 13, 1929.—Cultures were prepared with frozen and unfrozen tissue as described in the previous experiment. From the respective mixtures 2 cc. were placed in each of 4 Carrel flasks. The titer of virus at this time: frozen = 1:1,000; normal or unfrozen = 1:1,000.

Feb. 16.—10 bits of tissue were removed respectively from each of 2 Carrel flasks containing unfrozen (N) tissue and from each of 2 flasks with frozen (F) kidney, washed in Ringer's solution, and subcultured in plasma and embryo extract. The results of the subcultures were as follows:

    N1: 8 showed growth of cells, while 2 did not.
    N2: 5   "   "   " "   " 5 "   "
    F2: 10   "   no growth of cells.
    F3: 10   "   "   "   "   " "

Feb. 18.—To test again the viability of cells, 20 subcultures were made from N3 and N4 respectively and 10 from F1 and F2 respectively. The results of the tests were as follows:

    N3: 14 showed growth of cells, while 6 did not.
    N4: 15   "   "   " "   " 5 "   "
    F1: 10   "   no growth of cells.
    F2: 10   "   "   "   "   " "

Titration of virus from 2 Carrel flasks containing unfrozen (N) tissue and from 2 containers with frozen (F) kidney resulted as follows: N1 = 1:1,000, N3 = 1:100,000, F3 = 0, F4 = 0.

From the results of Experiment II it is evident that certain cells are able to survive for at least 5 days in a mixture of serum and Tyrode's solution. It also appears that vaccine virus survived or multiplied in the presence of living cells while it ceased to be active in the cultures prepared with tissues killed by freezing and thawing.

Experiment III

Feb. 25, 1929.—The vaccine virus used in this experiment was obtained from N3 of Experiment II. It had been diluted ten times and stored on ice for 7 days. As previously described, cultures were prepared with frozen and unfrozen minced kidney tissue. Prior to distributing the cultures in flasks, the following controls were set up on mica coverslips:

    10 cultures of frozen tissue in plasma and embryo extract.
    10   "   "   "   "   "   serum and Tyrode's solution.
    10   "   "   unfrozen tissue in plasma and embryo extract.
    10   "   "   "   "   "   " serum and Tyrode's solution.

Subsequent examinations of the above controls revealed the following facts: None of the frozen tissues showed growth; 9 of the unfrozen tissues in plasma and embryo extract presented signs of growth; 5 of the unfrozen tissues in serum and Tyrode's solution showed evidences either of definite cell growth (Fig. 3), of wandering out of surviving round cells, or of beginning giant cell formation (Fig. 4) through cell apposition.

March 2.—30 pieces of unfrozen tissue were taken from each of 2 Carrel flasks (N1 and N3) and 10 of frozen were removed from each of 2 containers (F1 and F2). To test the viability of cells, these bits of tissue were washed in Ringer's solutions and subcultured in plasma and embryo extract. The results of the tests were as follows:

> N1: 10 cultures showed growth, while 20 did not.
> N3: 29   "   "   "   "   1 "   "
> F1: 10   "   "   no growth.
> F2: 10   "   "   "   "

At the beginning of the experiment, titration of the virus resulted in no vaccinal reactions in the rabbit. After incubation at 37°C. for 5 days the contents of the flasks were examined for the presence of vaccine virus. None was found.

The virus used in this experiment was culture virus from Experiment II. It had been diluted and stored on ice for 7 days. During that time it had ceased to be active. This experience coincides with the findings of Eagles and McClean (6) who have had difficulty in preserving culture virus. The results of Experiment III indicate that cells are not only able to survive but may at times multiply in a mixture of serum and Tyrode's solution.

DISCUSSION

The results of the experiments described above are in agreement with those obtained by other workers who found that tissues killed by freezing and thawing failed to support *in vitro* the multiplication of vaccine virus. No evidence was secured to cast doubt upon Maitland's observations concerning the increase of vaccine virus in a medium consisting of minced fresh normal kidney tissue suspended in a mixture of serum (1 part) and Tyrode's solution (2 parts). In this medium, however, which Maitland considered not to be a tissue culture and in which he thought autolysis of the cells to be extensive within 3 days, it was possible to show that many cells remain viable for at least 5 days. This was accomplished by subculturing bits of the

tissue in a favorable medium of plasma and embryo extract. Furthermore, it was found that a medium of serum and Tyrode's solution is capable at times of supporting multiplication of certain cells (Fig. 3).

From the work here presented it appears that the increase of vaccine virus obtained by Maitland did not occur in the absence of living cells. Nevertheless, Maitland has made a definite contribution to the study of viruses in that he has found a medium for the easy cultivation *in vitro* of vaccine virus and other infectious agents of a similar nature (7).

SUMMARY

Cells survive for at least 5 days and at times are capable of multiplying in a mixture of serum and Tyrode's solution used by Maitland for the cultivation *in vitro* of vaccine virus.

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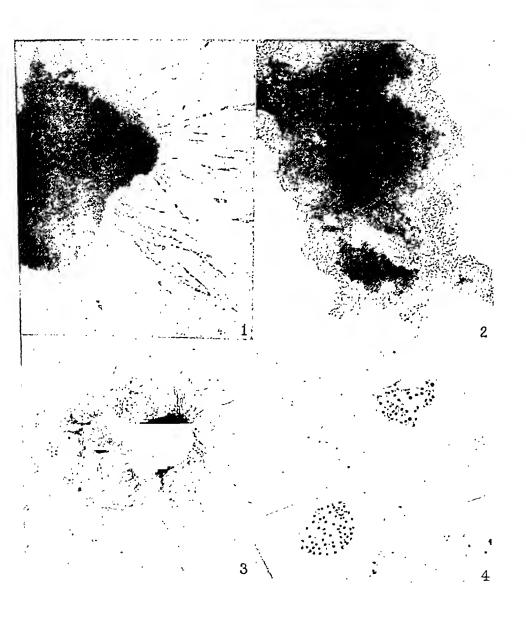
EXPLANATION OF PLATE 7

FIG. 1. A small piece of kidney tissue removed from a 3-day Maitland culture and subcultured in plasma and embryo extract. Note growth of cells. × about 95.

FIG. 2. Tissue treated similarly to that in Fig. 1 with the exception that it was

frozen and thawed before being used in a Maitland culture.   Note absence of cell growth.   × about 95.

FIG. 3. Fresh kidney tissue cultured 4 days in a mixture of serum and Tyrode's solution on a mica coverslip.   Note growth of cells.   Fixed preparation stained with Delafield's hematoxylin.   × about 85.

FIG. 4. Giant cells formed by cell apposition in a medium of serum and Tyrode's solution.   Fixed preparation stained with Delafield's hematoxylin.   × about 85.

STUDIES OF TISSUE MAINTENANCE

I. The Changes with Diminished Blood Bulk

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PLATE 8

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The present paper is the first of a series dealing with the service rendered to the tissues by the blood under various conditions. As indices to such service we have utilized the extravascular spread of easily recognizable, innocuous materials thrown into the blood stream. For the purpose of the work here reported certain highly diffusible vital dyes have been employed.

It goes without saying that the multifarious activities of the blood in relation to the tissues cannot be adequately comprehended through observations on the passage from and into the vessels of any single substance or set of substances. But one can at least obtain in this way a knowledge of the general problem in some of its quantitative aspects. Most of the information thus far accumulated concerning it is inferential in nature, being the outcome of observations on rates of blood and lymph flow, on the relative abundance of capillaries in different organs, on capillary pressures, and the state of the local circulation as determined by direct observation. Hooker, Krogh, Richards and others have made studies of the small blood-vessels which illumine the general field; and some investigators have followed directly the diffusion from or into individual capillaries. Our aim has been to determine what the blood does under pathological conditions for the various organs of the body as a whole.

The Choice of Materials

The peculiarities of living cells as manifested in what they reject or accept, and accepting, utilize, secrete, or store, renders the problem of tissue maintenance highly diverse. But it should be possible to

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find out in a general way whether the circulation is adequately serving the cells in bringing materials to them and away from them, irrespective of what the cells do with these materials. Susbstances that are let pass by the capillaries must, of course, be employed. True, these will be let pass in varying degrees. But it would seem to be a general law that diffusible, non-toxic "acid" stains penetrate the capillary wall at approximately the same relative rates as when they spread through gelatin (1). If a number that differ widely in this latter respect are selected for animal test it should be possible to gain an insight into how the tissues are served with materials normal to them. At first thought one would say that normal substances should be used. But not only are many of these subjected to change or removal through cell activities, with result that the gradient of permeability is altered locally, but their situation and quantity cannot be gauged with the eye as it can in the case of vital stains of intense color. The best of these stains have special affinities and are stored or excreted in ways that alter interpretations. But with the recognition of such peculiarities, errors due to them can be ruled out. The monographs of Schulemann (1) and of von Möllendorff (2) give one access to a large series of vital dyes. From amongst them and from other sources it should be possible to select test-substances covering the diffusion range of most materials normally purveyed to the cells, with exception of the gases.

The recent history of vital staining is an instructive one, illustrating as it does how rapidly the uses of a scientific tool of wide applicability can become stereotyped as result of success with it in a special field. Ehrlich brought vital staining to modern attention, making significant observations on the nervous system with the aid of methylene blue about thirty years ago (3). But with the subsequent discovery that certain of the poorly diffusible dyes are taken up and stored within living cells, interest turned almost wholly in the direction of the disclosures thus made possible. Not very often since, despite the immense gamut of available dyes, has diffuse vital staining been employed, and then, with a few noteworthy exceptions, for highly specialized purposes. Furthermore the range of dyes utilized in the study of cell-storage has of late narrowed instead of broadening, the mass of investigations nowadays being conducted with one or another of but a few slightly diffusible stains. An observation of the earlier investigators should be mentioned because of its bearing on our own problem, namely that dye storage is especially abundant in the eye muscles, diaphragm, and heart,—whence these workers inferred the existence of an especially great fluid interchange in the organs mentioned (4). Recently Okuneff (5) and Kusnetzowski (6), using the same

criterion, have concluded that much more dye reaches the cells of regions that are inflamed or heated than is the normal case.

For the present work highly diffusible stains devoid of confusing tissue affinities have been selected—Patent Blue V, brom phenol blue, phenol red and sodium indigotate. In enlargement of some of the observations india ink has been pressed into service. Ordinarily we have followed only the distribution of the dyes from blood to tissues, not their subsequent removal from the latter.

Patent Blue V is an intense stain of great diffusibility (7).* Within a few seconds after it has entered the blood stream white animals become brilliantly blue; and by pressing out the blood from the tissues the extravascular situation of the dye can readily be demonstrated. To obtain data on its rate of diffusion into the various organs is difficult, so quickly does it enter most of them. One can always tell, though, where it is and where it is not; and it has the great advantage of rendering visible the walls of the arterioles. Unanesthetized rabbits injected with $1\frac{1}{2}$ cc. per kilo of a warmed, unbuffered 8 per cent solution of Patent Blue V (Hoechst), of pH 6. approximately, manifest no symptoms whatever; and etherized ones show no disturbances of heart beat or blood pressure that are observable with the kymograph, other than those fleeting ones produced by a similar quantity of salt solution. The dye retains its color within the organism. More than three-fourths of it can be recovered as such from the urine of the first twenty-four hours, and most of the rest later on.

Brom phenol blue, somewhat less diffusible and leaving the blood not quite so rapidly, is almost as intense a vital stain. Its range as indicator lies too far to the acid side for the blood and tissue reactions to affect its hue. For our purposes it has proved well-nigh ideal, being devoid, when properly purified†, of action on blood vessels or heart, and diffusing at such rate that the stages in its distribution

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* A correlation of the diffusibilities of the dyes *in vivo* and *in vitro* is being carried out in our laboratory by Dr. Frederick Smith who will report upon them later. It will suffice here to state that a watery solution of Patent Blue V diffuses through a porous glass disc of the sort employed for diffusion measurements by Northrop and Anson (*J. Gen. Physiol.*, 1929, **12**, 541) about as quickly as does dextrose. When the pores of the disc have been filled with gelatin it passes only about one fourth as rapidly as dextrose.

† As ordinarily sold for pH determinations brom phenol blue often contains contaminants affecting the blood pressure. Hynson, Westcott and Dunning have most kindly made for us a purification of their product, which has proved innocuous and devoid of cardiac or vascular effects in cats and rabbits.

can be followed in animals killed at brief intervals after injection.* Furthermore its color so dominates over that intrinsic to the tissues that direct comparisons of degrees of staining become possible. It is best employed in 4 per cent solution, and for intense staining a somewhat greater fluid bulk than of Patent Blue V must be injected intravenously, 3 cc. per kilo of cat or rabbit.† Our usual technic, as in the case of other stains, has been to inject the warmed dye solution during the course of one minute. By the end of this time the body surface is already an even, intense blue; and the failure of the tissues to change color when the blood is driven from them by pressure shows that the dye has already largely passed from the vessels. Neither then nor later are symptoms evident. Rabbits suddenly stained deep blue and placed on the floor sniff about inquiringly and when food is placed before them at once fall to. Three minutes after the dye injection the staining is nearly at a maximum throughout the animal, though accurate color comparisons show that it deepens slightly within another five minutes, remaining constant then for approximately 4 hours and gradually fading later. By next day the animal is practically decolorized, the dye lingering only where the circulation is poor, as in the cartilage of the ear which is still light blue, the tendons (paler blue), the sclerotics (faintly blue); or about a locus of special retention as in the case of the blue gall-bladder wall through which resorption is still taking place secondarily from bile blue-black with the dye excreted with it. At this time the blood plasma no longer is colored. The phthalein leaves the body chiefly by way of the kidney though a little escapes in the feces. As much as 94 per cent of the amount injected has been recovered in the urine of the first 48 hours. The liver takes much out of the blood at an early period, secreting it into the bile, but, as happens with so many other stains, it passes into the circulation again from the gall bladder and intestinal tract, and ultimately escapes in the urine after all. No evidence has been obtained of the least decolorization of the dye within the organism. If any occurs it is negligible.

Phenol red diffuses twice as fast as does brom phenol blue‡ and somewhat more rapidly than Patent Blue V. Much of what has just been said applies to it. Because of the current utilization of it in renal tests it is readily available in pure

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* Dr. Smith has found it to pass through the porous disc of Northrop and Anson at a speed slightly more than half that of Patent Blue V irrespective of whether the disc has been filled with gelatin or not.

† We are indebeted to Dr. MacInnes for freezing point determinations which show that the solution we have employed is isotonic with 0.92 per cent NaCl, and to Dr. Mirsky for observations with the glass electrode which show the pH to be approximately 7.24 at 37°C. The method of preparing the solution has been given in a previous paper.

‡ Unpublished observations of Dr. Smith.

form; and the injection of a freshly made, warmed, isotonic 4 per cent solution of it at pH 7.4, to the amount of $3\frac{3}{4}$ cc. per kilo, the optimal quantity for staining, causes no symptoms or cardiac or vascular manifestations that a corresponding amount of Ringer's solution would not elicit. It can be used to study blood service to the connective tissue and the other relatively alkaline matrix tissues (cartilage, fascia and tendon(8)) where it appears ruddy, but not for observations on the muscles and viscera, since in them it assumes various shades of orange and yellow that are not readily perceived and evaluated when only a small amount of the dye is present.

Sodium indigotate is poorly soluble at best, only to 2 per cent in water at body temperature; and as much as $7\frac{1}{2}$ cc. per kilo must be injected if the color native to the organs is to be drowned in blue. It is reduced to indigo white in many of the tissues, though it turns blue again on exposure to air; and it is rapidly excreted by liver and kidneys. For all these reasons it has proved unsatisfactory in the study of blood service, although it can be used in confirmation of certain phenomena.

General Procedure

Rabbits and cats have been used for most of the work, and light ether as the anesthetic when one has been necessary. Many of the rabbits have been un-anesthetized. To begin with, the normal distribution to the tissues of brom phenol blue was ascertained, and a routine method of examination was worked out. In order that the spread of the dye to the superficies might be studied the fur was in many instances removed from a large part of the trunk and thighs some days prior to the observations, the animal being guarded against chilling thereafter. Barium sulphide proved better for the purpose than shaving. Areas accidentally inflamed were rapidly discriminated by the special intensity of the staining. Ordinarily the animals were fasted 24 hours in order to avoid a digestive hyperemia of the gastro-intestinal tract (Bier); but they were allowed water. Etherized ones were kept on an electrically warmed pad, and were not stretched out but laid on the back or side, without ties. Tracheal cannulation ensured a more even anesthesia. The dye was injected into an ear vein of rabbits and into the basilic vein or internal saphenous of cats, these vessels frequently being cannulated for the purpose. Oiling the body surface brought out brilliantly the surface hues. Since the abrupt introduction of even as little as 1 cc. of fluid into the circulation of a large rabbit brings about compensatory readjustments (Tigerstedt) the dye solution was given gradually in the course of one minute, as already stated. Ordinarily three further minutes were let elapse and then the animal was killed, by cutting both carotids, or,—when no anesthetic had been used,—by decapitation at a blow. The complication of stained blood within the tissues was minimized by the rapid exsanguination. The autopsy was carried out at once and very rapidly, by two workers, with the animal on a slanting board, head down. The organs of special interest were first looked at, the order of

inspection being purposely varied to rule out the possibility of errors due to post-mortem diffusion of the dye. The intensity of the staining as viewed in the gross, was frequently recorded in terms of Ridgway's color standards (9) according to the method used in previous investigations (10). Save in special instances we have not concerned ourselves with the precise location of the dye within the tissues, the main point being that it should have left the circulation, have been served up to the cells, so to speak, irrespective of acceptance or rejection by them.

Not infrequently an amount of blood equivalent to that of dye was removed just prior to injection of the latter. But needless to say this proceeding merely complicated the issue. For not only must some vasoconstriction have been invoked by it, but the salt solution containing the dye must practically at once have been removed from circulation. Whatever the importance of these various factors they did not suffice to bring about differences in the picture. The animals as a group yielded consistent findings, irrespective of the stain employed.

The Staining in Normal Animals

The phenomena observed after the injection of brom phenol blue were essentially the same in rabbits and cats, and were unaffected by etherization. By the time the injection was completed the *hairless tip of the nose*, the *gums, conjunctivae* and *fauces* had stained deeply and evenly. The pads of the feet became blue only a little more slowly and less markedly. The *general body surface* took on color progressively and evenly except for intensifications where vessels were abundant, as over the heels and about the mammae. After three minutes the surface hue was brilliant (between "cadet blue" and "diva blue"—Ridgway), the stain lying in the connective tissue. The thin sheet of *voluntary muscle* coming away with the pelt was but palely blue, much paler than the external and internal oblique and the pectoral and leg muscles. These in turn were not nearly so well stained as the *diaphragm, intercostals* and *lingual muscles*, the differences being especially plain when muscle layers of the same thickness were compared. To the unaided eye the staining of *subcutaneous tissue* and muscles appeared diffuse, and this still held true when the animals were killed only a few seconds after the injection. For example the tissues of a rabbit killed within 15 seconds after an injection lasting 30 seconds appeared diffusely stained, as did those of a cat killed 15 seconds after an injection which had itself required 30 seconds. But in certain noteworthy instances of animals kept for the routine period of 3 minutes there were indications in the muscles of a latticing or transverse banding with blue, a phenomenon later found to be pronouncedly present when staining was done after the blood bulk had been reduced by bleeding or through the production of anhydremia (11).

The *connective tissue* and *fascia* were everywhere of a medium blue, the *cartilage,* —except for the rib cartilages which fail to stain,—a lighter blue, and so too with the *tendons* and the newer portions of *bone*. Old bone remained uncolored.

Though the supporting framework of the *adipose tissue* was well colored the fat itself did not stain, nor did the white matter of the *central nervous system*, the gray matter being dubiously tinged.   The *nerves* to the muscles were beautifully visible in grayish blue to their finest ramifications.   The *red bone marrow* appeared deeply colored, but the dye was localized to the blood content.   There was intense staining of the media of the larger *arteries* suggesting a special affinity, though the subsequent decolorization took place nearly if not quite as rapidly as elsewhere save in the aorta where it noticeably lagged.   The latticing and banding in the muscles mentioned above was not due to this vascular staining.   The walls of the *veins* stained poorly.   The fibrin of post-mortem blood clots stood out in deep blue.   Embolism and thrombosis were sought for but never found.

The tissues thus far described were only moderately blue as compared with most of the abdominal viscera.   These were so suffused with the dye as to afford a startling contrast.   The *intestines*, large and small, were a deep purplish blue, and so too with the *oesophagus* and *gall-bladder*.   The bladder bile of rabbits was already definitely blue after three minutes, but that of cats only later.   The peritoneal surface of the *stomach* appeared rather light blue, though finely stippled with darker blue points in the case of the cat; but when the organ had been slit open the mucosa and submucosa proved to be deeply stained, like the gut further down. It was the gastric muscularis into which relatively little of the dye had gone. Occasionally there were to be noted in it areas of local contraction which had not stained at all though the inner and outer layers of these areas had stained as well as ordinary.   In this connection the fact deserves mention that the segments of large intestine distended with fecal masses were as excellently stained as the empty, contracted regions between.   A notable example was furnished by the rabbit colon wherein fecal pellets are usually distributed at nearly regular intervals with a thinned wall over them and contracted gut between, like coarse beads on a thick, gristly string.   Such a colon when slit longitudinally, emptied and inspected between glass plates had the same color intensity everywhere.

So rapidly did stain pass into the *mesenteric lymphatics* that they were distended with deep purply-blue fluid within 15 seconds after the dye injection.   The glands at the root of the mesentery contained a similar fluid.   In view of the extremely rapid diffusion the viscera were ordinarily inspected first of all, often before the heart had stopped.

In the gross the *liver* appeared deeply and evenly colored, but Valentine knife sections disclosed minor variations in hue, the periphery of the lobules being more intensely colored than the center, and both appearing greenish as compared with the clear blue of the interlobular connective tissue.   In rabbits killed after three minutes some stain had already reached the bile.   The *spleen* was a more or less deep blue,—less when the organ was somewhat contracted.   The *kidney* cortex was dark blue, the medulla lighter, and brom phenol blue was present in the cortical tubules.   The *urinary bladder* was medium blue, irrespective of whether it was full or empty, the hue approximating that of the superficial connective tissue. The *omentum* of the cat was lighter.   That of the rabbit proved too filmy for useful

observations.   Both in omentum and *retroperitoneal* fat the stain was localized to
the connective tissue framework.   The *pancreas* appeared evenly and rather lightly
stained.   Here too the color was principally in the connective tissue.

The *ovaries* stained rather intensely, and so too with the medulla of the adrenals,
the cortex staining scarcely at all.   The *pregnant uterus* was deeply blue, but into
amniotic fluid and well-developed fetuses the dye had not penetrated during the
few minutes following the injection.

The *lungs* were evenly and lightly blue, and the *thymus* too.   The *heart wall*
appeared deep purple blue.   We have not attempted to determine how much of
the color was due to contained blood.

In significant contrast to the mesenteric *lymphatics and glands*, those of the
limbs contained fluid that was at most but palely blue.

Substantially identical findings were obtained with Patent Blue V, though no
banding or lattice work was disclosed in the muscles, so rapidly did the dye diffuse.
With sodium indigotate, the far greater intensity of the staining in the viscera was
readily demonstrated, but minor differences were not easily to be apprehended
owing to the reduction of the dye to a colorless form.

A number of the organs considered above will not be referred to again in the
course of the present paper, notably the heart, kidneys, thymus, ovaries and
adrenals.   We have described them merely to round out the picture.

In summary one can say that certain organs or tissues (brain,
lamellated bone) are entered practically not at all by the dyes of our
experiments, that others receive but little of them (cartilage, tendon,
new bone, nerves), others show them in considerable quantity (con-
nective tissue, urinary bladder, muscle, pancreas), while others yet
become so suffused as to constitute a group apart (liver, gall-bladder,
intestines, stomach).   The deep color of the spleen and red bone
marrow is deceptive, the dyes lying for the most part still in the blood
contained in these organs.   In not a few others they are localized
almost entirely to connective tissue scaffolding and interstitial fluid.
With the binocular microscope one can readily make out that the
epithelium of the gut is stained only faintly if at all, and that in
voluntary muscle the color lies in general between the fibres.   But,
as has already been stated, our object has been merely to determine
whether the dyes are purveyed to the cells, not whether they are taken
up.   The observations just recounted yielded a norm for the distribu-
tion from the circulation.

Certain affinities of brom phenol blue require recognition at this
time.   It has some affinity for the media of arteries, as also for medul-

lated nerves.   In common with many other dyes it is rapidly removed from the blood by liver and kidneys.   Möllendorff has correlated the rate of excretion of such dyes into the bile with their physical properties (12); yet the activities of the liver cells in secretion of them are still not wholly understood.   The failure of the phthalein to penetrate old lamellated bone is evidently due to the constitution of the latter. The failure of the brain to stain with vital dyes in general has never been satisfactorily explained.   The intense color of the diaphragm, intercostals and tongue as compared with the other skeletal muscles is understandable on the basis of their more abundant circulation (13).

The Technique of Depletion

When the blood bulk has been suddenly and considerably reduced, by bleedings or procedures causing anhydremia, profound alterations take place in the service rendered to the various organs by the circulation.   Some of the changes have been briefly described in a report on the local, extravascular acidoses arising out of the state of affairs (14). We shall here consider them more fully.

To deplete the cats and rabbits used in the present observations successive bleedings have been employed.   Since the circulatory alterations disclosed by the staining method are seen in pronounced form only when the blood bulk is not restored through readjustments within the body, the animals were fasted from 18 to 24 hours prior to experiment in order to lessen the utilization of fluid from the gut, as further, to avoid digestive hyperemia.   Usually they had access to water; but under the circumstances they drank little.   Those that were etherized and connected with the kymograph by way of the carotid were bled, either from this carotid by puncture of the rubber tubing just above the cannula, or preferably from an axillary artery cannulated for the purpose.   Rabbits were bled with the aid of local anesthesia (novocain) by a method which involves a preliminary operation under general anesthesia to bring the carotid to the surface, cannulate it, and place about it an elastic clamp.   The clamp was made from a short piece of rubber tubing about 3 mm. in outside diameter which was bent upon itself and tied so tightly at the bend that its limbs did not lie in parallel but sprung somewhat apart.   The contrivance was slipped about the artery, and the free ends of the tubing were pulled through a constricting ring of rubber formed from the segment of a larger, thick-walled tube.   To shut off the vessel the ring was rolled toward it along the pieces of tubing, the pressure of these latter upon each other sufficing for the purpose.   For bleeding the ring was rolled away.   There was an optimal position of the latter at which the vessel was held gently shut, to be opened merely by pressing the ends of the contrivance toward each other, thus

springing its sides apart.   Successive bleedings were readily carried out with the animal on its feet.

It was early found that a large depletion, especially in the absence of general anesthesia, was required to bring about deviations in blood service so pronounced that certain regions which ordinarily color well failed to stain.   No effort was made to determine the least loss of blood that would suffice for this purpose. When it was accomplished by a progressive anhydremia the blood pressure often varied little from the normal (15); but when bleeding was employed it usually fell to about 100 mm. Hg in cats, and 60 mm. Hg in rabbits, in the absence of any efforts to conserve it.   After a first hemorrhage, as is well known, fluid from the tissues enters the vessels; but this readjustment is ordinarily completed within a few minutes, and takes place to but a slight extent after later bleedings (16). To rule out its influence upon the findings, as also to permit conditions to become relatively stable, three or four bleedings were done in all, at ten to twenty minute intervals, and the dye injection was ordinarily deferred until twenty minutes after the last one.   Great care was taken that the depilated, depleted animals should not grow cold during the experiments.   The unanesthetized ones were kept in warm rooms, and those under ether were in addition placed on electrically warmed pads.   With the successive bleedings the skin and mucous membranes became pallid, and the superficial veins more or less collapsed.   Very occasionally an ill-defined surface mottling could be made out.

Ordinarily almost half, if not quite half, of the calculated blood volume (which is approximately 7.5 per cent in the cat, 5.5 per cent in the rabbit) was removed. The rabbits depleted with local anesthesia still kept their feet but the respirations were exaggerated.   That the alterations in the staining were not traceable to low blood pressure, as such, had been disclosed by the observations on anhydremic animals (17), and was now further shown by the fact that the longer the interval elapsing between the final bleeding and the injection the more pronounced were the deviations from the ordinary staining, although in the interval the pressure often tended to recover.   The same amount of dye per kilo was given as under normal conditions, and in the same way.   It sometimes caused a partial recovery of the blood pressure, but no symptoms.   As in the case of the controls, the animals were killed three minutes after the injection.

The Alterations after Reduction of the Blood Bulk

The phenomena occurring in the *superficial tissues*, in the pelt that is to say, of animals receiving brom phenol blue have been briefly described in a preceding paper.   The cat or rabbit turned blue more slowly than usual, the rate depending on the degree of depletion and how long after it the dye was injected. In extreme instances only a faint blue staining developed and this was limited to the regions where large vessels entered the skin.   In the majority of instances the staining was patchy, unstained areas being everywhere interspersed amidst others that became brightly and diffusely blue.   This patching proved in the

cat to be essentially similar to that already described for the rabbit and rat (18) but the white areas tended to be larger. They were irregular in outline, with serpiginous margins, frequently confluent, and varied in size according to the depletion conditions, the arrangement being sometimes of blue on a white ground and again of white on blue. In poorly marked instances there was merely a scattered sprinkling of small white spots on the blue expanse. The patching was especially well seen in well-nourished white cats with an underlying panniculus. In extreme instances, in which phenol red had been used instead of a blue dye, there took place a very gradual orange staining,—the color indicative of acidosis,—about the largest arteries entering the skin, while elsewhere the surface was unstained. The orange-red hue of the conjunctiva in such cases pointed to a blood acidosis. In less severely depleted animals the injection of the phthalein was followed by a brilliant mottling of red on white or white on red. Where the dye crept in later, at the edges of the unstained regions, it was seen to be orange.

Slight differences in local pressure sometimes exerted a great influence to determine the situation of patches in the depleted animal (19). Crouching rabbits often showed a broad, unstained strip along the ridge of the backbone and other large unstained areas over the bulge of the knees. It was necessary to allow for such localizations, as further for the influence of isolated masses of fat to make an overlying, thin, translucent skin appear poorly stained. Where errors of interpretation from these causes could be ruled out one saw that the patches were not only highly irregular in contour but without trace of symmetry. On reflecting the pelt the relation of them to the blood vessels could be made out; and the arrangement of the patching was found to be wholly independent of the vascular pattern visible in the gross, save in the extreme instances above mentioned in which only the tissue immediately about the largest vessels was stained. This independence was especially well to be seen where a number of arteries supplied the subcutaneous tissue in parallel, the lumbar vessels of the cat, for example. Here some of the arterial twigs ran to patches that were white, other corresponding ones to areas that were blue, and yet others to areas that were irregularly blue and white (Fig. 2).

The patching was not essentially dependent upon cooling of the skin, though frequently developing when it was cool. It appeared pronouncedly in one of our animals which was accidentally overheated on the pad; and it can be regularly elicited in rats submerged in oil at body temperature (20). If nothing occurred to relieve the depleted organism the white areas persisted for long periods, though tending gradually to diminish in size by a peripheral encroachment of the dye. When they were very large dye sometimes appeared secondarily at spots here and there within them. Obliteration of them occurred in both ways when the blood volume was restored by reinjection of the portion removed.

The *gums, nose tip, conjunctivae* and *mucous membranes of the mouth* colored promptly and deeply even when they had appeared absolutely bloodless prior to injection of the dye. They showed no patching, nor did the pads of the feet, which also stained rapidly but somewhat less well.

The *skeletal muscles* exhibited a remarkable reticulation or transverse banding with blue as has already been briefly recorded. And the pattern, unlike that of the skin, was regularly ordered, and did not vary in its dimensions with the degree of depletion. It could be studied directly under the binocular dissecting microscope in certain of the flat muscles (tibialis anticus, gracilis, pectorals, extensor longus digitorum) wherein it took the form of a transverse banding, and in others (as e.g. those of the abdominal wall) where a blue reticulum separated oval, unstained areas. To obtain specimens glass plates were slipped over and under the muscle which had been loosened from the tissues sufficiently for this purpose, and the attachments were then cut. The banding was now seen with the unaided eye to be discontinuous, consisting of many short blue segments displaced regularly a little to this side or that of the main axis of the band. When the muscle was thick (quadratus lumborum) the segments were frequently superimposed at different levels. The blue reticulum was likewise discontinuous. For the present it will suffice to say that the arrangement of the staining was determined by that of the vascular tree, and that it occurred about vessels of a special magnitude, several times larger than capillaries. The average length of the latter in rabbit muscle is 0.69 mm. (21), while the unstained regions between the blue bands (or blue reticulum) were more than 2.0 mm. across in this species, and even broader in cats.

Greater differences were evident in the staining of the various muscles than under normal circumstances. The cutaneous muscle layer did not color at all after severe depletion, and the sheet muscles of the abdomen but slightly as compared with the brilliantly banded quadratus lumborum and gracilis. Even these were pale in comparison with the diaphragm, intercostals and tongue muscles which were diffusely stained and almost if not quite as deep blue as ordinary. Sometimes the intercostals showed a slight relative pallor midway between the ribs; but the blue was uniformly distributed in diaphragm and tongue even when the animal had been sacrificed only a few seconds after injection.

The findings thus far described bear witness to pronounced and peculiar empoverishments of blood service. Not so with those in the *gastro-intestinal tract*. Here the staining appeared to have the same intensity as ordinary, and only on recording the hues in terms of Ridgway's book did one perceive it to be slightly less. As result of the fasting the duodenum and jejunum were wholly collapsed as a rule. The *intestines*, large and small, were always a diffuse, even, purply blue, and so too with the *gall-bladder* wall, *esophagus* and the mucosa of the *stomach*. In extreme instances the muscularis of this last organ showed an ill-defined, slight, pallid blotching but as a rule the coloration was diffuse. The staining was as good where the intestines were stretched over fecal masses as in the contracted lengths between. All this was true even when the animal was killed practically at once after the dye injection. Already the *lymphatics* of the mesentery and of the gall-bladder wall were distended with deep blue lymph.

The *liver* was, as in the normal animal, diffusely and deeply blue. The *pancreas*

was stained about as well as ordinary and showed no patching. The *urinary bladder*, on the other hand, was decidedly paler than in controls, often indeed almost unstained, but without any patching. And the condition of the spleen showed frequently that it had been shut off *in toto* from the circulation, even in etherized animals. Contracted and small the organ stood forth in vivid red against deep blue surroundings. When depletion had not been severe small spreading areas of blue were found scattered amidst the red of the surface exposed on section.

The *omentum* of cats presented a singular picture, being splotched with blue and white wherever it was thick enough for the presence or absence of staining to be made out. The distribution of the stain was irregular, as in the case of the skin, and independent of the vascular patterning visible in the gross. It was confined to the connective tissue. The unstained patches were often several centimeters in greatest diameter. In the retroperitoneal fat a similar splotching or marbling with blue was brilliantly evident. The omentum of the rabbit proved too filmy for satisfactory study.

A contrast to these evidences of ischemia was furnished by the *uterus* of cats far pregnant. The organ was found markedly and evenly stained despite the severest depletion, and the veins coming away from it contained much deeply colored blood although there was little elsewhere owing to the exsanguination at death. In this connection mention may be made of the fact that inflamed areas in the skin of depleted animals stained excellently.

The *kidneys* were diffusely blue, to the naked eye at least. *Tendon* and *cartilage* (ear, knee joint) colored somewhat less well than in controls, but there was no patching. The deep hue of the *red bone marrow* (legs, ribs) on the other hand showed that the dye had reached this tissue abundantly. The *lungs* stained lightly, as in controls, save at their wedge edges which were often wholly uncolored. The tissue at the apices was no paler than that elsewhere.

The lymphatics and glands of axilla and groin, in contrast to those draining the intestines, contained a fluid only faintly tinged with the dye.

These various findings were readily confirmed with Patent Blue V, and some of them with phenol red. The patching of the skin could be demonstrated with sodium indigotate, as also the profound staining of the gastro-intestinal tract, gall-bladder included.

In summary of the observations it can be said that under the circumstances of a markedly reduced blood volume certain of the organs continue to be well and evenly served by the circulation, whereas in others a pronounced patchy ischemia occurs. The conjunctiva, lips, gums, pharynx, oesophagus, stomach, intestines, gall-bladder and liver all become rapidly and deeply stained; and the dye appears in quantity in the lymph from the gut and gall-bladder, even

when the carotid pressure has been greatly lowered by the bleedings. Liver lymph was not studied.   The red bone marrow continues to be excellently served by the blood in animals at the extreme of depletion, and so too does the pregnant uterus.   The skin on the other hand and the voluntary muscles, with certain notable exceptions, are largely deprived of effective circulation.   The deprivation takes a singular form, regions showing it being interspersed amidst others in comparison excellently served by the blood as evidenced by the staining.   The size and number of the patches in the skin varies directly with the degree of depletion and the length of time it endures.   Their distribution is entirely unsymmetrical and they seem unrelated to the vascular patterning visible to the eye.   In the muscles on the other hand there is a regular disposition of unstained and stained regions, the latter situate about vessels of a special, and not inconsiderable magnitude. The unstained regions have a diameter several times greater than the length of the individual capillary.   Not all of the muscles suffer this neglect.   The diaphragm, intercostals and tongue muscles continue to be excellently supplied by the blood, as the depth and evenness of the staining attest.

Though blood service is remarkably well sustained in most of the abdominal organs there are certain significant exceptions.   The spleen is largely, sometimes entirely, deprived of circulation, as shown by the failure of dye to enter it, and the urinary bladder, lightly colored in the controls, is often practically unstained in the bled animals.   In the omentum pallid, ischemic regions are scattered irregularly amidst others well supplied from the blood as shown by their brilliant blue color.

Observations with India Ink

It has seemed important to determine the precise relation of the blood vessels to the ischemic patching just described.   For this purpose we have resorted to india ink injections.   A principal result of the work has been the recognition of how far the method falls short of demonstrating circulatory conditions within the body.

The distribution of india ink by the blood stream has been followed by a host of workers.   Krogh has made large use of it in his studies of capillary regulation. Our own observations with ink of the sort he employed (Pelikan Perl Tusch,

Günther Wagner), dialyzed against Ringer's solution and filtered in the way he describes (22), would seem to indicate that his findings were obtained in the face of serious technical draw-backs. When the amount of ink that Krogh employed is injected intravenously into a rabbit or a guinea-pig, and the vessels of the ear are watched under a microscope, one can perceive that the foreign particles tend to agglomerate into lumps as they are carried along. These lumps soon lodge here and there within or at the entrance to capillaries, effectually blocking them. Using such material one can be certain only that where it passes the vessels are open. There is, fortunately, no difficulty in demonstrating with it that many more such vessels are open in the diaphragm of the normal animal than in most of the other skeletal muscles, a phenomenon emphasized by Krogh. But to assure oneself that where the ink does not penetrate no circulation had existed is quite another matter. And this holds true even when an ink far better for the purpose is used, Higgins' American Drawing Ink (non-waterproof), dialyzed against Ringer's solution, filtered and centrifuged—the last two processes being unnecessary in our experience. On injection of this the particles can be seen to circulate separately during the brief period before they are removed from the blood by the sessile phagocytes.

Normal white animals receiving either Higgins' ink, or Pelikan Perl Tusch, become transiently gray. We had supposed that in depleted ones patchily but intensely colored with brom phenol blue, injected ink particles would pass into the stained regions of skin and muscle in sufficient quantity for histological recognition if the animals were killed while they still circulated. But this did not prove to be the case. To all intents and purposes the particulate matter was shut off from the organs mentioned, and this proved to be the case as well in animals that had been merely depleted, not stained. The skin did not turn gray anywhere. The abdominal viscera on the other hand were dark with ink, except for the spleen which contained practically none. The liver was black with that which had been taken up by the Kupffer cells. In contrast to the pallid state of the skin generally, the tip of the nose, and the pads were gray with ink, lying doubtless within the arterio-venous anastomoses there known to exist (23, 24). The quantity injected was only $2\frac{1}{2}$ cc. per kilo, because of the need to avoid large increases of the blood bulk. Further findings will be detailed in a succeeding paper.

DISCUSSION

The method of the present work would seem to be validated by the disclosures it has yielded. The alterations we have observed in the

service rendered by the blood to the skin, muscles, and certain other organs of the depleted animal cannot be apprehended by ordinary laboratory procedures; nor are they demonstrable with india ink. In animals depleted by large hemorrhages a rapid and deep staining occurs in some regions (lips, gums, tip of the nose) which under such conditions in unstained animals appear wholly bloodless; and furthermore staining occurs in skin regions to which india ink particles are not carried by the blood. It seems probable that in some of these situations only a stained plasma may have circulated, red cells being removed by the "skimming" that Krogh first described. The great diffusibility of the dyes we employed proved in some ways a disadvantage; for they were so readily distributed that only drastic reductions in service to the tissues were recognizable with their aid. To appreciate less considerable changes it will be necessary to employ vital stains that do not leave the blood so rapidly.

Can it be said that where brom phenol blue failed to go all interchange between the blood and tissues had ceased? Scarcely. For Patent Blue V gradually penetrated where brom phenol blue did not. And carbon dioxide, which passes through the tissues with unexampled ease (25), reaches situations inaccessible to other substances. To all intents and purposes nevertheless the circulation had ceased to be effective in the skin areas which brom phenol blue or phenol red failed to penetrate. For an acidosis developed in such areas, one referable to the local accumulation of acid metabolites (26).

In appraising our results a first question is, how far they were conditioned by special affinities of the dyes employed? By employing several of highly different constitution we have tried to minimize this factor; but in another and better way it has been proven unimportant, namely by following the process of decolorization. Those organs which stain most rapidly and deeply with brom phenol blue are, with exception of the liver and kidneys,—which actively excrete the dye,—precisely those which lose color soonest, as could scarcely happen were it fixed upon them as result of a special affinity. The gastro-intestinal tract and the diaphragm become colorless long before the skin does. Brom phenol blue stains the media of arteries with a special intensity; but even here decolorization does not lag notably.

A principal alteration in blood service after hemorrhage is a peripheral vascular shut-down, the blood supply to the viscera being maintained at the expense of that to the superficial tissues and the muscles. This readjustment has long been recognized as one of the means whereby the vital forces are conserved in individuals "bled white." Other changes disclosed by the staining method impress one with their purposefulness. Not all of the abdominal organs continue to be well served by the blood. Those which are essential, and which can be safely neglected,—the omentum, urinary bladder and spleen,—are neglected. By contrast the whole gastrointestinal tract —from which alone help can come to the organism under natural conditions,—continues to be well served. Special mention may be made of the maintenance of the circulation to the gall-bladder, since the realization is recent that a very active resorption takes place through the walls of the organ into the blood and lymph. Even the esophagus and fauces are well served, and the gums and lips are much better maintained than is the skin. The red bone marrow continues to be excellently supplied with blood, though situated within limbs that are for the rest largely deprived of it.

The amount of blood which would be conserved to the organism were there complete ischemia of the skin is but slight (2 to 3 per cent) (27), but the saving of heat is far more considerable. And the cooling of the neglected tissues lessens the formation of waste products within them. In the voluntary muscles, large, enduringly bloodless patches do not develop as in the skin, but there is a regular arrangement of smaller ones into which a certain amount of diffusion gradually occurs, as shown by the findings with Patent Blue V. But not all of the muscles suffer in this way. Those which are essential to respiration (diaphragm, intercostals) and to swallowing (tongue muscles) continue to be well served by the blood. The question whether this is true because these muscles go on working need not be taken up at the moment, though the fact may be mentioned that the muscles of the tongue continue to be well served from the blood, as shown by a diffuse, deep staining, even when the organ lies flaccid in animals anesthetized through a tracheal cannula.

Under normal circumstances much more stain has been found to pass from the blood into the tissues of the gastro-intestinal tract than into

the skin and muscles. Numerous reasons for this can be thought of. The amount of blood passing in a unit of time through the skin and resting muscles is many times less than through the portal circulation. Capillaries are far more numerous in the viscera while furthermore a great proportion of those existing in the skin and resting muscles are ordinarily shut. There is an active flow of lymph from the blood into the mesenteric lymphatics, but practically none into the lymphatics of resting muscles (28, 29), whence it follows that a dye circulating in the blood penetrates into the muscles only by diffusion, whereas the process of distribution to the tissues of the gut is actively aided by a streaming of fluid out of the vessels. Furthermore the distribution to the gut is aided by a high capillary blood pressure, and the barrier offered by the capillary wall itself is imperfect, as shown by the presence of blood proteins in lymph collected from the mesenteric channels (30). Considering all this one cannot wonder that the stomach and intestines showed a specially intense and rapid staining with vital dyes.

From what is known of the physiological readjustments which take place in the bled animal one might expect the staining in the gastro-intestinal tract to disclose wide deviations from the normal. The capillaries of the region are known to be actively contractile; and a narrowing of the portal channels through vaso-constriction is deemed one of the most important compensatory changes occurring when the blood volume is diminished (31, 32). Even losses of blood which do not suffice to lower the arterial pressure cause some blanching of the intestines (Starling); and when such a lowering has taken place the rate of formation of lymph in the gut and liver is markedly reduced (33). Not a few functional conditions have been described in which blanching of the intestines was so great that the tissue seemed to all intents and purposes bloodless. For every *a priori* reason, then, except the teleologic, one might suppose that blood service to the gut after hemorrhage would be greatly lessened, perhaps to the extent of ceasing in some regions. But the dye experiments showed quite another state of affairs to prevail. Staining of the gut, gall-bladder and liver was always deep, though the animal was at the extreme of depletion and was killed but a few seconds after the dye injection; and the staining was diffuse save occasionally in the muscularis of the

stomach where an ill-defined blotching could be made out. The mesenteric lymphatics were always distended with deep blue fluid. The pancreas likewise was colored as usual. One must conclude that the compensatory constriction was never so great as to interfere seriously with blood service to the digestive organs.

Mention has been made of the fact that the staining of the intestine where it was stretched over fecal masses was of precisely the same intensity as in the empty, contracted segments lying between. The pressure condition where feces distend the gut is probably much like that in the full bladder, of which Sherrington remarks (34) that it "enfolds its contents in the same light grip whether these contents be ample or little." Owings, McIntosh, Stone and Weinberg (35) have ascertained that in normal dogs the greatest intraintestinal pressure is equivalent to only 2–4 cm. of water.

The amount of dye injected into the depleted animals was the same as in normal ones. Since the blood volume had been reduced by nearly half in most instances, it follows that the dye circulated in unusually great concentration. The intensity with which the abdominal viscera stained despite the untoward conditions, must be attributed in considerable part to this cause. But it will not explain the patching of the omentum, the more or less complete failure of the urinary bladder to stain, or the neglected state of the skin and muscles, which, so far as they received blood at all, received the same sort as did the viscera.

Starling has pointed out that normally absorption goes on from the digestive tract irrespective of whether there is a body need for the materials absorbed, the sole recourse of the organism being a regulation through the excretory organs which remove at an appropriate pace that which has been taken into the body willy-nilly (36). It is plain from our findings that even when the blood bulk has been diminished to the limit of tolerance, adequate circulatory conditions are maintained for absorption from the gut, a process which frequently acts to sustain life. Robertson and Bock have proved that salt solution introduced into the intestine is far more effective in permanently restoring the blood pressure of human beings after hemorrhage than when it is thrown directly into the circulation or injected into the tissues (37). The reason for this is not yet clear.

The dyes we have used are rapidly excreted into the bile; and this

of course makes for a deep staining of the liver. Even within so brief a period as three minutes after the injection of brom phenol blue or Patent Blue V, much dye had reached the finer bile ducts. The dyes yielded no evidence that after hemorrhage some hepatic regions were better off than others. Yet the vaso-motor regulation within the liver is far from negligible (38); and a "stroking reaction" can be elicited on the surface of the organ, like the cutaneous one so much studied (39). In view of all this one of us has made a special study of the character of the hepatic blood service after hemorrhage. The results are detailed in the paper which follows.

The mackerel-sky or lattice work staining in the muscles of bled animals was obviously related to the arrangement of the vascular tree. That it was largely dependent upon contraction of the vessels, was shown by experiments in which vaso-constriction was prevented from occurring in the muscles of a leg by cutting the nerves to it just prior to injection of the dye. In the muscles of a limb so treated staining took place diffusely whereas in those of the control leg the usual mackerel-sky patterning was found. Our many experiments of the sort will be described in detail on another occasion. Not infrequently normal, stained cats and rabbits exhibited traces of the patterning here or there, more especially in the gracilis and quadratus lumborum. It may with good reason be attributed to that partial vaso-constriction on which maintenance of the normal blood pressure depends, but there are other important conditioning factors as will be shown subsequently. In not a few cases, just before the introduction of the dye an equivalent amount of blood was removed from the circulation. There was no more pronounced patterning in such instances.

The factors responsible for the patching of the skin are less readily to be explained. They too are dealt with in a subsequent paper. The possibility has already been ruled out (40) that the patching depends on a differing intrinsic permeability of vessels of like magnitude supplying tissue of the same general sort, a difference becoming effective only when the blood flow has been cut down by vaso-constriction.

Some foreshadowings of the changes we have observed in the service to the tissues after depletion can be found in previous work. Meek

and Eyster, watching directly the circulation in the dog's ear, noted that after a considerable loss of blood there suddenly occurs an active contraction of the capillaries and small venules (41). They suggest that possibly "when the circulation is at the breaking point as it is when the bleeding equals 2 per cent of the body weight the venules and capillaries are constricted in widespread areas." The pronounced restriction of blood service occurring in the skin and muscles under such conditions is not uniform, as our experiments show, some regions being still fairly served while neighboring ones are wholly passed by. Langley (42) noted that the circulation continues through a few arterioles in the muscles of the frog after hemorrhages severe enough to stop it in the generality. He believed that a similar state of affairs would be found to exist in mammals. Gesell and Moyle (43), who ascertained the volume flow through the muscles of dogs repeatedly bled, found that at late stages of the gradual depletion it was reduced to an extent out of all proportion to the drop in blood pressure.

The vascular shut down in the spleen after hemorrhage is no new phenomenon (44). One may contrast therewith the state of affairs in the red bone marrow as disclosed by our experiments. Not only does dye still reach this tissue in quantity but india ink does as well.

The conditions as concerns blood service to the kidney under pathological conditions are complicated and we have made no attempts to study the organ. Richards states that relatively few kidney glomeruli are open to the circulation in frogs that have lost blood but that the number can be greatly increased by restoring the blood bulk (45).

<div align="center">SUMMARY</div>

The spread through the living animal of various highly diffusible dyes has been utilized as an indicator of the ability of the circulation to serve the tissues under various conditions. The method is direct and searching. Blood service to the viscera, as demonstrated by it, is normally far more profuse than to the skin and muscles, for evident physiological reasons. After hemorrhages which greatly reduce the blood bulk service to the viscera is in general still well maintained even though the animal be *in extremis*. However great the compensatory contraction of the splanchnic vessels may be,—and physiologists have long supposed it to be very great,—it certainly does not suffice to

hinder blood service anywhere in the digestive tract. On the other hand the service to certain unessential abdominal organs (spleen, omentum, urinary bladder) is cut off in large part or wholly; and in comparison with the essential viscera, the skin and most of the skeletal muscles of the bled animal are largely deprived of circulation. This neglect takes a curious form, some regions being still fairly served by the blood while others next them are no longer ministered to. In the skin the areas served, or not served, are highly irregular but are to some extent determined in situation by local pressure factors. Within the muscles the neglect is orderly in arrangement and is largely referable to compensatory vaso-constriction. Certain of the muscles, those used in respiration and in swallowing, furnish significant exceptions to the general rule, being excellently served despite the serious general state. The red bone marrow of the depleted organism continues to be well served by the blood even though situated in limbs that are, for the rest, almost devoid of a circulation. The pregnant uterus also is excellently maintained despite the serious general state.

The changes are such as would tend to conserve the forces of the depleted organism and to contribute to its recovery.

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EXPLANATION OF PLATE 8

Fig. 1. Skin of the side of a white cat injected with brom phenol blue after reduction of the blood volume by repeated bleedings under ether. The brilliant blue and white mottling is only moderately well shown in the photograph; yet the contrast is sufficiently great to suggest that the white patches were raised above the blue, as was not the real case. The hair had been removed by shaving.

Fig. 2. Reflected skin of the same cat showing three parallel distributions to the subcutaneous tissue from the series of lumbar vessels. Some of the blue patches in this tissue occupy the regions supplied by one or another of the secondary arterial branchings; but of others this is not true. Three branches that correspond in situation are indicated by arrows. Two of them run to colorless patches whereas the third enters tissue that is heavily stained.

STUDIES OF TISSUE MAINTENANCE

II. The Service to the Liver and Digestive Tract after Hemorrhage

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PLATE 9

The service rendered to the various organs by the blood can be gauged by the distribution to them of diffusible vital stains. In a previous study by the method, there have been observed certain well-marked alterations occurring after the blood bulk has been reduced by hemorrhage. The service to the organs immediately essential to life and to recovery (heart, lungs, respiratory muscles, alimentary canal, liver, red bone marrow) was maintained at the expense of that to certain others (skin, voluntary muscles in general, spleen, urinary bladder). But these latter organs,—with the exception of the urinary bladder and sometimes of the spleen,—did not suffer to an equal degree throughout, regions in which the circulation was ineffective being interspersed amidst others still served by the blood, as shown by the stain that entered the tissues. It has seemed possible that a similar, if less pronounced, patchy neglect might after all have been present in the splanchnic viscera that appeared well and uniformly served, a neglect masked by rapid diffusion of the dyes, or by a rapidly intermitting circulation, now to this region and again to that. The present work was undertaken to cover the point thus brought up.

One does not have to look far in the literature to find that the vessels of the gut and liver are sufficiently contractile to render the assumption reasonable that a functional ischemia may exist in these tissues on occasion.

Mall showed in 1892 that during stimulation of the splanchnics the blood flow from the systemic arteries to the portal vein is greatly cut down. There occurs

also an active contraction of the portal twigs within the liver (1). These observations on vasomotor activities within the hepatic tissue have been repeatedly confirmed (2). In anaphylaxis of the dog an interference with the circulation through a spasm of the small hepatic vessels leads to the characteristic drop in blood pressure (3). Mautner and Pick (4) have adduced experiments to show that the liver of cats possesses a mechanism whereby contractile spasm of the liver capillaries is produced. According to these authors the action of epinephrin or barium chloride upon the vessels causes obstruction to the blood flow through the cat's liver as not through that of rabbits. Ebbecke brought about a capillary dilatation with edema on stroking the hepatic surface, which he deemed comparable with the cutaneous stroking phenomenon that has of late been so much studied (5).

The fact is well attested that contraction of the portal bed through vaso-constriction is largely responsible for the maintenance of blood pressure after hemorrhage. Krogh has advanced evidence on this point (6). Starling states that a loss of blood too slight to reduce the general blood pressure will cause blanching of the intestines (7). In experiments upon some lower forms an extreme blanching has been elicited in various ways. Bayliss states, in summary of general knowledge, that the innervation of the abdominal viscera is predominantly vaso-constrictor whereas that of the peripheral tissues is vaso-dilator (8). And yet, if one may judge by observations from our laboratory, the vascular readjustment which takes place after large hemorrhages in cats and rabbits, cuts down and may almost abolish the effective circulation to the periphery while interfering but little with that to the viscera.

Method

For the present work india ink was mainly employed. It was mixed with a 4 per cent solution of brom phenol blue or preceded by an injection of the stain, or of phenol red, when information was desired of the state of blood service to the skin and muscles. Though this is brilliantly disclosed with the dyes it cannot be with ink, which, in animals that have been bled, frequently fails entirely to reach the tissues mentioned. Higgins' American Drawing Ink (non-waterproof) was employed. For injections during life this is far superior to the Pelikan Tinte (Günther Wagner) so much used by Krogh, since the particles circulate separately, not in agglomerates of various size. The ink was dialyzed against Ringer's solution for some days and was then both filtered and centrifugalized, processes which separate out few if any particles. 2½ cc. per kilo was introduced at body temperature into a vein, during the course of one minute. There followed only the rise in blood pressure which an equivalent amount of salt solution would have produced. The animals employed were rats, rabbits and cats. In some of the early work general anesthesia was induced with urethane, but this proved unsatisfactory for reasons to be given later. Light ether was employed with all of the cats and they were bled from a cannulated axillary vein. Rabbits can be

repeatedly bled from the carotid under a local anesthetic (9), but most of those employed were etherized. Kymograph records of the blood pressure were taken in many cases. The rats were bled from the carotid while under the influence of urethane or ether.

To learn the distribution of the ink in the stomach and intestines direct inspection during life was necessary; for needless to say much of it might be forced here and there through post-mortem contraction of the vessels. So rapidly are the ink particles removed from circulation by phagocytes sessile within various organs that the inspection has to be carried out within the first minutes after the injection. For these reasons the animals used for the study of the gut were under general anesthesia; and in order to rule out the effects of exposure, the abdomen was opened only when the time had come to look at the organs. In the case of the liver the state of affairs could be studied at leisure after the animal had been killed,—by exsanguination from the carotids, or by decapitation in the case of unanesthetized rabbits; for the ink is so rapidly taken out of the blood by the Kupffer cells that the amount of phagocytosis will serve as an index to blood service. But in order to rule out a possible post-mortem distribution of the ink, with such phagocytosis as result, it was essential to remove the liver from the body immediately upon death. Livers thus treated empty themselves of blood as they do not when they have been left in situ for even a few minutes with the large vessels uncut (10). In the present work the capillaries regularly proved empty. Microscopic preparations were obtained with the Valentine knife or the freezing microtome, and cleared in glycerine. If there was brom phenol blue in the organ, the complication of its intense blue hue was done away with by placing the sections in a solution possessing that slight degree of acidity necessary to turn the phthalein yellow.

The Maintenance of the Liver

In a series of preliminary experiments urethanized rats were bled repeatedly from a carotid; injected with ink; killed after various brief periods of time; and compared with controls merely receiving ink. It was a surprise to find that under these circumstances the spleens of the depleted rats contained as much ink as those of the controls; for Barcroft has proved that the spleen contracts after hemorrhage. Our finding was traced to the urethane, which acts to prevent contraction of the spleen, as Henning showed (11). When the rats had been bled under a local anesthetic, almost no ink reached the spleen. It was everywhere equally distributed to the liver lobuli, irrespective of the character of the anesthesia; but in the bled animals the peripheral Kupffer cells contained vastly more than did the central ones,—which was not the case in controls. In rabbits that were depleted under light ether a pronounced splenic contraction occurred, as proved both

by the appearance of the organ and the almost complete failure of ink to enter it. The rabbit liver on the other hand showed an abundant and even distribution of ink to all of the lobuli, but within these units the same pronounced divergence from the normal was observed as in rats. The peripheral Kupffer cells were swollen with phagocyted ink particles, while those further in had less and less the nearer they were to the center. Identical findings were obtained when the bleedings had been carried out with the help of local anesthesia.

In all of the foregoing instances depletion was purposely carried far, about half of the blood being taken as a rule. Sometimes 20 minutes had elapsed between the last bleeding and the ink injection, sometimes a less period, the plan being not to exhaust the activity of the vaso-motor center. The unanesthetized rabbits maintained the ordinary crouching posture. In every instance in which brom phenol blue had been mixed with the ink a pronounced patchy ischemia of the peripheral tissues was disclosed.

Identical findings were obtained in cats bled and injected under light ether, save for the fact that the ink was almost evenly distributed to the phagocytes within the hepatic lobuli, just as in the controls. Only when the depleted cats were sacrificed within a few seconds after the ink injection, with result that but little had been phagocyted, was more visible in the peripheral Kupffer cells.

The Service Rendered by the Hepatic Artery

The existence of two sources of hepatic blood renders uncertain the interpretation of the foregoing observations. That the two vascular trees supplying the hepatic tissue differ in their vaso-motor responses is known (12); and it might well be that the effects of a contraction of portal radicles here and there would be masked by a freer flow from the end arteries, or vice versa. For this reason observations were made on the distribution of ink after diversion of the portal stream from a part or all of the liver.

In some etherized cats and rabbits the branch of the portal vein running to the "main liver"—the mass lying between stomach and diaphragm—was tied, the result being that all of the portal blood was diverted through the "lobe mass"— the right posterior and caudate lobes. This abrupt change in the path of the blood is well tolerated by the organism, as an abundant experience has shown (13); and

in the course of the present work it has been observed to have no effect on the carotid pressure. Needless to say the necessary operation was performed with the least possible trauma and exposure of the viscera, only a short segment of the vein being dissected free for ligation.* The hepatic artery, in special, was not touched; and it was seen by direct inspection to continue to beat strongly. The abdomen was then closed with a running suture in two layers. After 2 to 18 minutes had been permitted to elapse, for purposes of readjustment within the organism, the bleedings were carried out as usual, and ink injected. When the animal was killed a little later the lobe mass was found coal black with phagocyted ink particles whereas the main liver was of a light or dark brown. In both portions of the organ the ink had been evenly distributed to the lobuli. To control the effectiveness of the ligation milk was injected under pressure into the portal vein below the tie. It flowed only into the lobe mass.

One of the animals, a rabbit, had been vitally stained with phenol red just prior to the portal diversion and its body surface had colored a uniform red. After the bleedings, at the time of the ink injection, the surface had become pronouncedly patched with buff upon red, plain evidence that there existed in the superficial tissues local areas of outlying acidosis, such as result from an ineffective circulation.

In some additional experiments (on rabbits) a preliminary operation was performed to induce a development of portal collaterals by partial obstruction of the flow to the liver, after Drury's modification of Moskowitz' method (14). Some weeks later when the collaterals were well developed the portal vein was completely tied off under ether. There resulted no alteration in the carotid blood pressure. After a brief interval to allow for readjustments the animal was depleted by successive bleedings according to the usual method, and ink, or ink and brom phenol blue, was injected. On sacrifice it was found that the effective blood pressure within the liver had been so greatly lowered by depleting the blood bulk that the slight pressure exerted on the organ by the stomach or ribs of the prone animal had sufficed to prevent the ink from entering certain regions. Elsewhere, though, it had been distributed evenly to the lobuli by the hepatic artery. The milk test showed that the portal stream had been wholly diverted from the liver.

In a final experiment the portal vein in a normal male cat weighing 3025 gm. was tied off, with result, of course, that the animal gradually bled into its own splanchnic vessels, the blood accumulating in the portal regions back of the ligature. The abdomen was closed as usual. The carotid blood pressure sank progressively and within 8 minutes the mucous membranes of the mouth, previously pink, had become pallid. After 18 minutes the animal was bled 20 cc. from the carotid cannula to hasten the depletion. After 35 minutes the carotid blood pressure was very low, having fallen from 150 mm. Hg prior to ligature to 40 mm. Hg. Now

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* The separation from its sheath of the portal vein of the cat or rabbit is a nice procedure, best accomplished after longitudinal incision of the sheath, by pressure with a pledget of cotton or gauze held in the grip of a mosquito forceps.

8 cc. of ink was injected intravenously during 35 seconds. The skin failed to color with it at all, but the mucous membranes of the mouth became gray in patches. 19 minutes later both carotids were cut. But little blood came away, nearly all having collected in the portal viscera, behind the ligature. The liver was slaty black with ink which had been evenly distributed to the lobuli by way of the hepatic artery. There was slightly more in the peripheral Kupffer cells than near the center. The milk test showed that the ligature had been occluding.

These experiments demonstrate that arterial blood is uniformly distributed within the liver even when the organism is at the extreme of depletion.

The Service Rendered by the Portal Vein

The literature on contraction of the portal radicles within the liver has already been reviewed. There is much to indicate that it exerts an important regulatory influence on the blood supply to the heart, notably after hemorrhage. The following tests were designed to show whether under such circumstances some parts of the hepatic tissue are served by the blood and others neglected.

In etherized cats and rabbits the hepatic artery was dissected free and ligated, at a single point in the earlier experiments, in the later at two points, above and below the origin of the A. gastro-duodenalis, in order to prevent all flow through collaterals. Two ligatures were placed at each situation, the abdomen was closed as usual, and bleedings and injection were carried out in the ordinary way. It was found, after the bleedings, that the intrahepatic blood pressure had been so greatly lowered that the slight pressure of the ribs sufficed to prevent the blood-laden ink from entering the tissue lying next them. Elsewhere, though, it was regularly distributed to the lobuli. The milk test showed that the ligatures had wholly cut off the arterial flow.

Again no evidence was encountered of a patchy maintenance of the hepatic tissue in animals with a reduced blood bulk. Some little arterial blood may have reached the liver by way of the diaphragm. The supply of arterial blood from this source is so small in the rabbit that necrosis of the liver and death regularly follow ligation of the hepatic artery. In the dog the supply is sufficient to avert this catastrophe.

The Service Rendered to the Intestines

When white animals (rats, rabbits and cats) are injected with ink after marked reduction of the blood bulk the body surface does not

become ashy gray as in normal controls. So few ink particles reach the skin and muscles that the regions of total ischemia in these tissues cannot be discriminated with their aid. As the foregoing experiments on the liver have sufficiently shown, they circulate in abundance through the portal bed. Special care had been taken in these experiments not to expose the intestines, in order to avoid vaso-dilatation due to trauma. The short incision necessary to tie one or the other of the hepatic vessels had been made high up to the left of the mid-line where only the upper surface of the stomach and the under surface of the liver came into view. Nevertheless a dilation of the capillaries of the gastro-intestinal tract sufficient to let ink through into the portal vein might have been an indirect result of the operative procedure. To learn whether in the absence of any such stimulus these vessels let ink through, as further to find whether they are patent everywhere, some animals were not laparotomized until after ink had been placed in circulation. The examination was made during the brief interval before the particles had been taken out of the blood; and heed was given only to the condition when the gut was first exposed. The animals had been fasted to rule out all possibility of the digestive hyperemia described by Bier. The stomach and small intestines of the cats were empty. In rabbits the stomach and large bowel were still distended with roughage after several days fasting.

Always it was found that despite the preliminary depletion the intestines and stomach were uniformly gray with circulating ink; and the blood of the portal trunk black. Such vascular contraction as may have taken place in readjustment for the blood loss had nowhere closed off the capillaries to such extent that ink particles could not course through them; and there was no sign of a patchy distribution of the material. The findings confirmed those with diffusible dyes (15). It can be concluded that such compensatory contraction of the small vessels of the alimentary canal as may occur after hemorrhage does not anywhere even nearly shut these vessels, and that the service rendered by the blood to the tissues is far less affected as a whole than is that to the skin and muscles.

Contractility of the Vessels of the Alimentary Tract

That the failure of the small vessels of the alimentary tract to undergo an occlusive contraction after hemorrhage is not due to any

inherent inability to contract has been shown by tests with epinephrin and pituitrin. After one of these has been given intravenously the blood supply to certain portions of the bowel may be so completely cut off that highly diffusible dyes fail to stain them. Service to the liver, though, as judged by this criterion, is still well and evenly maintained.

A sufficient amount of 1–1000 solution of suprarenin (Metz) (0.05 cc. per kilo, diluted with an equal part of 0.9 per cent saline) was injected into a vein of rabbits or cats to cause a great rise in the carotid blood pressure, and while this rise still endured brom phenol blue in the quantity usual for vital staining (16) was run into the circulation. As a rule a little of the suprarenin solution was mixed with it, the object being to sustain the high blood pressure. To avoid the possibility of cardiac default under the conditions the dye was injected more slowly than usual, in the course sometimes of as much as 2¼ minutes. In rabbits the blood pressure rise was succeeded by an irregular fall toward the previous level throughout the subsequent period of 3 to 4 minutes before the animal was killed. In cats on the other hand vagal inhibition of the heart beat caused the pressure to drop before it had risen very far, and frequently brought it below the previous normal. This, of course, did not mean that vaso-constriction had been relaxed. At the time when the carotids of the anesthetized rabbits were cut they were hugely distended, their diameter being often twice the normal,—clear evidence of a peripheral obstruction to blood flow.

In both the species used the service rendered by the blood to the skin and muscles was greatly interfered with by the action of epinephrin, as shown by the slow, slight, and irregular staining. The small intestine, colon, and rectum on the other hand stained as well as normally, becoming deep blue within so short a time as a minute after the dye injection. The liver and gall bladder were also rendered diffusely blue and the phthalein promptly appeared in the bile. On the other hand the mucosa of the stomach of rabbits showed large, pallid, serpiginous areas on a blue ground. These contained neither dye nor blood. They had no obvious relation to the vascular arrangement, and the overlying muscularis was as well stained as ordinary. The caecum showed irregular unstained patches involving the whole thickness of the wall. These likewise were unrelated to the patterning of the vessels. In cats such evidence of visceral ischemia was wholly lacking, although in skin, muscles, and omentum it was pronounced. The cat spleens were not contracted whereas those of the rabbits were.

Very singular was the condition of the mesenteric lymphatics of the rabbits. Their contents became colored with blue as rapidly and deeply as ordinary; but after this had occurred the epinephrin must have induced an irregular spasm of the lymph channels themselves, the consequence being that they had the appearance of broken segments of blue thread, or,—in cases in which they were larger, with bulgings between the valves,—of irregularly contracted blue·tapeworms. So great had the spasm been in two animals that the channels had ruptured in numerous places near the gut and there were spreading extravasations of blue lymph. Falta and Priestley (17) have made injections comparable to mine into dogs and have studied the vascular state by direct inspection of the organs. They found the intestines as a whole to be greatly blanched, and inferred that the circulation is diverted elsewhere. In my animals only the stomach and large intestine manifested any ischemia and in them its distribution was patchy. The interference with service to the gastric mucosa may have been attributable, in part at least, to contraction of the muscularis mucosae. Adrenalin stimulates this layer as it does not the muscularis proper (18). The ischemia involving the entire wall of the caecum can only be explained on the basis of vascular contraction.

Pituitrin yielded far more pronounced findings.

Two preparations were employed, Pituitrin (Obstetrical), Park, Davis and Co. and Infundin (Burroughs Welcome and Co.). As in the case of epinephrin, a preliminary intravenous injection was made to bring about vaso-constriction, and when a pronounced rise of the carotid blood pressure showed that this had taken place in the etherized animals brom phenol blue or Patent Blue V was run into the blood stream,—very slowly, else the heart failed and death occurred. For the pituitrin had caused an unexampled rise in the general blood pressure. At the time when the carotids were cut in order to kill the animal they were found distended to double the ordinary diameter. In two unanesthetized rabbits in which the blood pressure was not ascertained, the same injection procedures were successfully followed and with the same effects, as disclosed at autopsy. The alterations in the service rendered to the tissues were profound. The skin and voluntary muscles in general stained almost not at all during the routine 3 minute period after the dye injection (Figs. 1 and 2). The liver and gall bladder on the other hand were intensely and evenly stained and so too with the esophagus and small intestine. The stomach on the other hand was sometimes almost unstained throughout (Fig. 2), and again exhibited serpiginous pallid patches which were or were not limited to the mucosa. The large intestine sometimes showed

patching throughout and again was completely unstained.   The omentum was marbled with stain or completely unstained.   The spleen was sometimes blue, and again was contracted and not stained.   The lymphatics deriving from the stained gut held deep blue fluid, whereas the contents of those from the pallid large bowel was colorless.   No great contraction of any of these channels was discernible.

Both the preparations employed caused an interruption of service to the stomach and large bowel as shown by the more or less complete failure of these viscera to stain.   The small intestine was by contrast deeply colored.   The "Pituitrin" caused vigorous peristalsis with expulsion of feces, whereas the "Infundin" did not elicit this activity, the feces being retained, and the stomach and bowel, examined before life had wholly ceased, showing no abnormal contraction. It is conceivable that in some situations contraction of the muscular layer of the gut may have reinforced vascular contraction, with result in the ischemia encountered.   But staining with the dyes employed is so extraordinarily swift that even a temporary relaxation of the muscle, as during peristalsis, would have sufficed to permit it, especially since the blood pressure was abnormally high.   The inference is that the ischemia was due to the same cause as that in the skin and muscles, namely to vascular contraction.

It is possible that had the blood volume of the animals been reduced by bleeding just prior to the experiments, or had an amount of pituitrin been given which caused a less intense vascular contraction in the peripheral tissues some diminution in blood service to the small intestine might have come to light.   The bloodless state of the skin and muscles has already been commented upon.   With the narrowing of the circulatory channels everywhere the blood and the dye added thereto would inevitably be forced to the regions of least resistance, quite irrespective of whether the vessels within these regions,—which might well have been intestinal,—tended to contract.   In our experiments the lungs can scarcely have served as reservoirs for blood forced out from elsewhere.   They were less stained even than ordinary. Room for some of the excess blood was obtained in the large vessels, as the distended condition of the carotids proved; and some was held in the dilated heart.   But in the circumstances of the case, with even the vessels of the stomach and large intestine closed to some extent,

much blood may have been foisted, so to speak, upon the small intestines and liver.   Our interest did not lie in determining whether this was actually the case, but merely in the demonstration that the blood vessels in some parts of the gut, at least, are capable of such contraction as to prevent service to the tissues by way of them.

<div align="center">COMMENT</div>

The experiments demonstrate that such vaso-constriction as may take place in the alimentary canal and liver to compensate for a lessened blood bulk never goes so far as to prevent the blood from rendering service to these organs.   Not only do diffusible dyes continue to be rapidly distributed to them, but their capillaries fail to offer any obstacle to the passage of india ink particles.   In contrast to the state of affairs in the skin and muscles, where well served regions are interspersed amidst others wholly neglected, the service to the hepatic parenchyma and the wall of the gut is evenly distributed. The maintenance of this uniformity was put to an extreme test in the case of the liver by cutting off the venous or arterial blood supply to the organ in animals with blood volume largely reduced.   Under these circumstances the remaining stream to the parenchyma flowed with so little force that the slight pressure of ribs and stomach sufficed to turn it aside here and there, with result in patches of total ischemia.   Had there been any local differences in the degree of constriction of the small vessels elsewhere in the organ surely these should have been evident in differences in the staining.   None were observed, the lobuli being all served to precisely the same extent.   Yet the existence of a vaso-motor mechanism within the liver is a fact that cannot be gainsaid.   One must suppose it to be so admirably balanced in the healthy animal that all parts of the liver share equally in the blood; for otherwise local hypertrophy, and a concomitant atrophy, would occur (19). Mall observed that a colored mass injected into the freshly extirpated organ reaches the capillaries of the lobules everywhere at the same moment, and this no matter whether introduced by hepatic vein, portal vein or hepatic artery (20).   The present experiments prove that the regulation continues undisturbed during the compensatory readjustments which follow reduction of the general or local blood bulk. In view of all these facts the active hyperemia followed by edema

(stroking phenomenon) which Ebbecke elicited on the liver surface must be thought of as a distinctly pathological manifestation.

It had seemed barely possible that under the circumstances of a general depletion the liver lobuli might be intermittently supplied with blood, the rate of intermittence being so rapid that staining with a vital dye or the scattering of india ink to the Kupffer cells wouldsoon appear to have been broadcast. To settle the point thus raised some animals were killed only a few seconds after injection of the test material. Even in them the findings showed an even distribution of blood to the individual lobuli, though within these units one could see that the cells first reached by the stain or ink particles, namely those at the lobular periphery, shared in these substances more abundantly than those nearer the central vein. In the case of normal animals receiving ink,—which substance can be followed with special ease,—this difference disappeared after a few minutes, the Kupffer cells all appearing to have shared equally in the particulate matter, even when the amount was small. So too with cats which had been bled. In bled rabbits, on the contrary, there was a startling change in the distribution, almost all the ink particles being held in the peripheral Kupffer cells, which were swollen and black with the engorged material. A number of reasons for this suggest themselves, amongst them that of the known differences in the intrahepatic vascular responses of the rabbit and cat (21).

The maintenance of a well distributed and effective blood service to the gastro-intestinal tract after hemorrhage is necessary if the gut is to function in aid of recovery. Conditions would be doubly disastrous were service greatly cut down. Yet in view of all that has been written on intestinal blanching and compensatory vaso-constriction within the portal system after hemorrhage one might have expected some regions of local ischemia at the least. In this connection the demonstration of a previous paper may be recalled, that blood service is often still going on in tissues which, to the eye, appear wholly bloodless.

Bayliss has made the generalization that the vaso-motors to the viscera are predominantly vaso-constrictor in type, and those to the periphery vaso-dilator. While this [7] generalization holds for the ordinary circumstances of life, certainly when the blood bulk is reduced the compensatory vaso-constriction is far more effective at the periphery than in the liver and alimentary canal.

SUMMARY

The vascular readjustments in compensation for a greatly reduced blood bulk affect the service rendered by the blood to the gastrointestinal tract and liver far less than they do that to the skin and muscles. Into these latter tissues india ink is carried almost not at all, whereas it circulates in quantity through the capillaries of the bowel and liver. Evidently vaso-constriction is much less effective in these viscera. Nowhere in them does one find a patchy ischemia like that so wide-spread in the peripheral tissues. Blood service is maintained to the same extent everywhere throughout the liver even when one of its two sources (hepatic artery or portal vein) is obstructed, and the intrahepatic blood pressure brought very low.

A pronounced patchy ischemia of the stomach and large bowel can be induced by intravenous injection into normal animals of sufficient epinephrin to cause the systemic blood pressure to mount to an abnormally high level. Pituitrin used in the same way has a greater effect; blood service to the organs mentioned may be completely abolished by means of it. In both instances, though, service to the small gut and liver is still excellently and evenly maintained.

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EXPLANATION OF PLATE 9

Fig. 1. Organs of an unanesthetized white rabbit receiving brom phenol blue intravenously and decapitated two minutes later.

Fig. 2. Organs of a white rabbit treated in the same way but receiving pituitrin four minutes prior to the stain.

It will be seen that the skin of the right side of the pituitrin animal is unstained whereas that of the control was colored, and though the subcutaneous muscle (seen attached to the skin on the left side) was stained that of the control was much more deeply so. The stomach of the pituitrin rabbit though mottled with dye was largely unstained, whereas the stomach of the control was intensely colored. The small intestines of both animals were stained to the same great degree, but the caecum of the pituitrin animal showed some splotchy, partial pallor. The blue of the animals photographed so poorly that the brilliant character of the differences is far from evident.

Fig. 3. A more pronounced instance of pituitrin ischemia. The stomach of the rabbit injected with pituitrin followed by brom phenol blue has stained almost not at all, and so too with the descending colon,—which is recognizable by its content of fecal pellets. The small intestine, on the other hand, is intensely colored.

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THE EFFECT OF UNILATERAL NEPHRECTOMY ON THE TOTAL NUMBER OF OPEN GLOMERULI IN THE RABBIT

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PLATES 10 AND 11

Karsner, Bunker and Grabfield (1) and others have demonstrated that removal of one-half the kidney substance in the dog results in a transient renal insufficiency with complete recovery in 1 to 2 days. It is apparent that the opposite kidney compensates for the loss. The majority of the published papers summarized by Hinman (2), deal with blood and urine findings and late hypertrophic changes. So far as we are aware, there is no work to explain the immediate compensation occurring after unilateral nephrectomy.

Through the work of Richards and his collaborators (3) the conception of periodic functioning of glomeruli has been placed on a firm foundation. Hayman and Starr (4) have demonstrated that in general, kidney volume, renal blood flow and urine elimination vary in direct proportion to the total number of open glomeruli.

Method

The technique of Hayman and Starr (4) with some modifications was followed. Rabbits were used throughout. As indicated in the protocols ether anesthesia was employed in some, and in others section of the lumbar spine by the technique of Ecker (5).

In control animals the lower abdomen was opened, the intestines wrapped in a warm towel, the aorta isolated and clamped at the bifurcation and just below the renal vessels, a long slender glass cannula introduced into the aorta between the clamps, the upper clamp removed and the cannula inserted so its tip was at the level of the diaphragm. The outside diameter of the cannula was approximately

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one half of the internal diameter of the aorta. With the cannula in position, the superior mesenteric artery and coeliac axis were clamped and 0.75 cc. of 3 per cent solution of Janus Green B (Coleman and Bell) in 0.9 per cent NaCl solution injected under moderate pressure. The time elapsed during the injection averaged 5 seconds. Within 10 seconds the left renal artery was ligated and the animal killed by a blow on the head or by ether. In some cases the left renal circulation was irrigated with saline and filled with 5 per cent ammonium molybdate. In others the left kidney was removed, cut in thin slices and placed in the molybdate solution. After death of the animal the right kidney was injected by the method of Nelson (6).

In experimental animals, the right kidney with as long a pedicle as possible was removed through a lumbar incision. It was then injected supravitally with Janus Green for a determination of the total number of glomeruli. After a variable period, the left kidney was stained intravitally by the procedure outlined above for control animals.

In the animals on which a sham operation was performed, the kidney region was exposed by lumbar incision, the kidney freed from its bed, the pedicle exposed, the whole replaced and in some cases a suspension suture placed in the capsule. After variable periods these animals were subjected to the same procedure of intravital staining as the control and experimental group.

The whole procedure may be summed up as follows: One kidney is injected supravitally to determine the total number of glomeruli in one kidney of this animal; the opposite kidney is injected intravitally for a determination of the number of glomeruli which are in active circulation during a period of 5 seconds under certain experimental conditions; and division of the latter by the former gives the percentage of open glomeruli.

The counts were made after the general technique of Vimtrup (7). The cortex and medulla are separated and the weight of the entire cortex secured. From the cortex, 6 to 10 small samples are removed, pooled and accurately weighed. The total weight of these samples varied from one-tenth to one-sixth of the entire cortex. From the number of glomeruli in these samples of known weight, the total is secured by estimation. In some cases, microscopic sections were also prepared to determine the completeness of injection in supravital injections and to check the percentage injected by the intravital method.

Results

As shown in Table I, in seven adult rabbits, the percentage of open glomeruli varied from 44 per cent to 78 per cent with an average of 63 per cent. In general those in which ether was used as an anesthetic have slightly more than those with fractured spine anesthesia.

After unilateral nephrectomy the percentage was increased to 91 to 99 per cent with an average of 95 per cent (Table II). This

increase appeared in less than 24 hours and continued until at least 10 days after nephrectomy.

TABLE I

Normal Controls

Rabbit No.	Supravital		Intravital		Per cent open	Anesthetic
	Kidney No.	Count	Kidney No.	Count		
1	45	179,767	46	79,640	44	Section spine
2	51	185,769	50	101,193	54	Section spine
3	69	158,400	70	124,410	78	Ether
4	71	147,734	72	110,670	74	Section spine
5	76	152,238	75	100,702	67	Section spine
6	78	138,406	77	92,386	67	Section spine
7	80	149,134	79	98,210	66	Section spine
Average..					63.5	

TABLE II

Experimental

Rabbit No.	Supravital		Interval in days after nephrectomy	Intravital		Per cent open
	Kidney No.	Count		Kidney No.	Count	
8	93	102,300	1	98	99,700	97
9	94	132,750	2	99	130,111	98
10	95	170,930	3	100	165,453	92
11	96	135,670	5	101	122,500	90
12	97	181,000	10	102	178,420	98
13*	54	87,350	1	56	80,222	92
14*	55	111,508	2	58	101,538	91
15	61	172,331	3	63	169,210	98
16*	57	68,620	5	60	67,752	98
17*	62	96,250	10	64	95,499	99
18	126	150,364	1	106	156,217	100
19	127	154,782	2	107	149,167	97

* Young animals, 5 to 7 months of age; remainder over 1 year of age.

The animals subjected to a sham operation (Table III) do not differ markedly from the normal controls. The open glomeruli vary from 51 per cent to 75 per cent with an average of 64 per cent.

DISCUSSION

There are certain points in the technique which need further comment, in order to validate the results.

Janus Green B is known to be a powerful vaso-constrictor and it is possible that it might cause such constriction that the results are not those present in life. Hayman and Starr (4) used India ink and obtained the same figures. Further, their results with salt and with caffeine, as well as the results here described following unilateral nephrectomy, prove that 90 to 100 per cent may be open with the same experimental technique.

TABLE III

Sham Operation

Rabbit No.	Supravital		Interval in days after operation	Intravital		Per cent open
	Kidney No.	Count		Kidney No.	Count	
23	118	161,208	1	119	82,344	51
24	104	150,100	1	103	102,614	68
25	120	158,316	2	121	93,562	59
26	122	122,488	5	123	86,920	71
27	124	135,728	10	125	100,786	75
Average...						64

The percentage figures depend on the assumption that the two kidneys of any one animal each contain approximately the same number of glomeruli. In addition to the proof offered by Haymann and Starr we add the results in two rabbits.

Rabbit No.	Left	Right
30	145,200	152,328
31	165,160	160,209

We also have unpublished observations that this is true in man and rats. The variation is rarely over 5 per cent. Further proof is adduced by the agreement between the counting method and the study of sections with determination of the relative number stained.

The accuracy of the weight estimation method for determination of the total number of glomeruli may be open to some doubt. We be-

lieve it to be accurate to within 10 per cent. Vimtrup (7) has offered evidence to support this view. Work soon to be published from this laboratory on the total number of glomeruli in the kidney of man and animals will further support the accuracy of the method.

The distribution of the dye in intravitally stained normal kidneys is peculiar. Never are the stained glomeruli distributed evenly throughout the cortex. As noted by Hayman and Starr, often the peripheral glomeruli are not stained while the more central are all stained. In other cases there are small to large areas corresponding to the distribution of an interlobular or interlobar artery in which very few glomeruli are stained. In selecting the specimens for counting, such areas must be taken into consideration and a proportionate amount of them contributed to the pooled specimen.

Richards and Schmidt (3) have noted that an increase in the number of open glomeruli is associated with an increase in the number of open loops of any one glomerulus. In general, we have found this to be true (compare Fig. 3 and Fig. 4). In the active kidney, more glomerular loops are stained and those that are stained are more definite and larger.

The results themselves are unequivocal and need little discussion. The results on the sham operation animals prove that the operation itself is not the cause of the increase. Experiments are under way to contribute evidence on the cause of the increase.

SUMMARY

1. Under the experimental conditions employed, from 44 to 78 per cent of the glomeruli of the normal rabbit kidney contain circulating blood at any one moment.

2. After unilateral nephrectomy the number of glomeruli in the remaining kidney, which contain circulating blood, is increased to 91 to 99 per cent.

3. Compensation for the removal of one kidney is accomplished during the first 10 days at least, by an increase of the number of open glomeruli in the opposite kidney.

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EXPLANATION OF PLATES

PLATE 10

FIG. 1. A teased preparation of cortex injected by Janus Green B, to show the definition of the glomeruli. Such preparations are used in counting. × 8.5.

FIG. 2. Section of kidney supravitally stained. × 110.

PLATE 11

FIG. 3. Section of normal kidney intravitally stained with Janus Green B. Note the unstained and partially stained glomeruli. Compare with Fig. 4. × 90.

FIG. 4. Section of kidney from animal after unilateral nephrectomy intravitally stained with Janus Green B. Note the absence of unstained glomeruli and the more complete staining of the glomerular loops. Compare with Fig. 3. × 90.
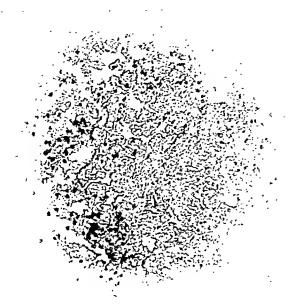
Fig. 1

FIG. 3

FIG. 4
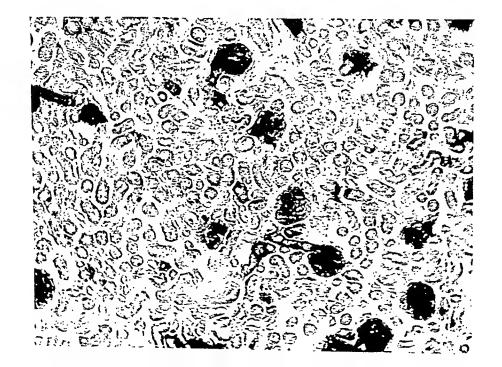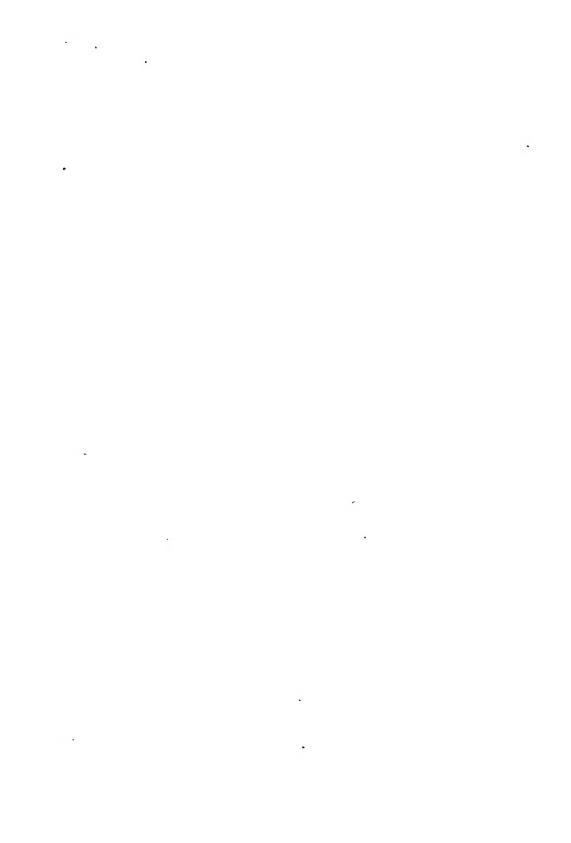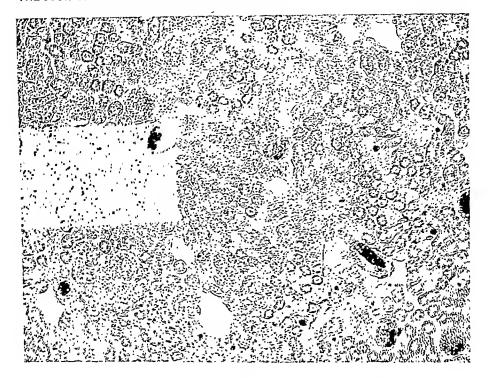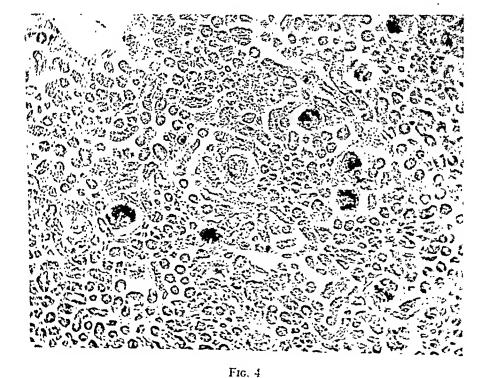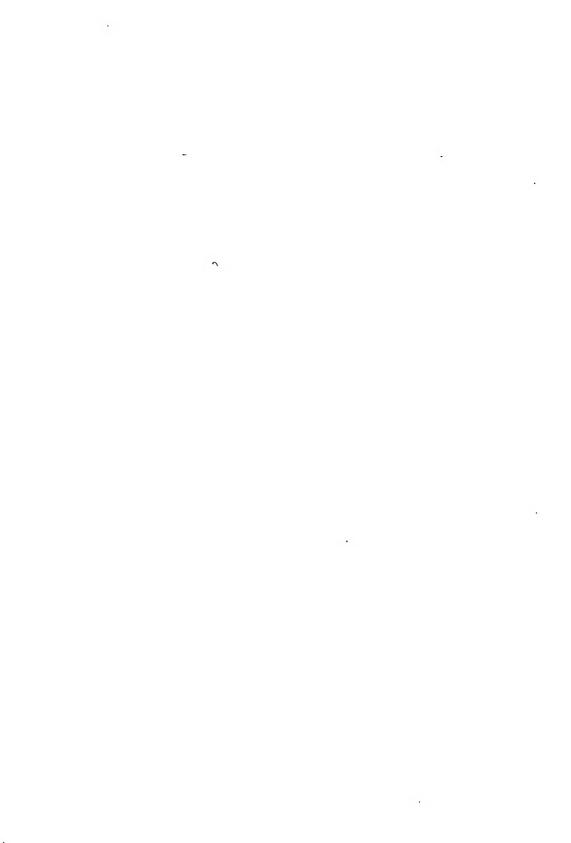
(Moore and Lukianoff: Unilateral nephrectomy in the rabbit)

A STUDY OF PNEUMONIA IN A RURAL AREA IN SOUTHERN ALABAMA

By W. G. SMILLIE, M.D. and E. L. CALDWELL

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The present study was undertaken to determine certain basic facts in relation to pneumonia in a rural community in the South; it covers a period of 6 months, October to April, 1927–1928. Our field laboratory[1] was established in Andalusia, Alabama, a town of about 4,000 inhabitants, the center of a rather sparsely settled rural community where farming and lumbering are the chief industries. The area covered in the study comprises approximately a thousand square miles, and has a population of about 35,000, the great majority of whom are white.

Types of Pneumococci Isolated

Fifty-eight cases of pneumonia were seen in the Andalusia area during the course of the study. A summary showing the types of pneumococci isolated from these cases is given in Table I.

The striking feature of this series of cases is the small number of fixed types of pneumococci that were isolated. Pneumococci of the heterogeneous Type IV group were the prevailing organisms.

Severity of the Disease

In Table II we have classified the cases of pneumonias according to their severity as correlated with the type of pneumococcus isolated.

Lobar pneumonia takes a much heavier toll in the northern than in the southern United States. In 1925, for example, the death rates

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[1] We are greatly indebted to Col. C. A. Reasoner of the U. S. Army Medical Service, for help and advice in selecting equipment for our field laboratory.

per 100,000 from all types of pneumonia in whites and negroes for certain typical states were as follows:

	White	Colored
New York	105.3	367.4
Massachusetts	116.4	230.5
Pennsylvania	116.7	366.4
Alabama	81.4	135.1

TABLE I

Types of Pneumococci Isolated Compared with Data from New York City
(Monograph 7, Rock. Inst.)

Pneumococcus Type	No. of cases	Alabama	New York City
		per cent	*per cent*
I	7	12.3	33
II	0	0.0	31
IIx	2	3.5	—
III	1	1.7	12
IV	40	68.5	24
Pneumococcus not isolated	8*	14.0	—

* Two of this group had a pneumonia due to staphylococcus, one following a periostitis—the other a case of senile dementia, while one case had a hemolytic streptococcus in pneumonia following an automobile accident. The remaining five were children from 1 to 8 years of age, from whom it was difficult to get sputum.   They were presumably pneumococcus pneumonias, Type IV.

TABLE II

Severity of the Disease

Type	Mild	Moderate	Severe	Fatal
I	0	3	4	0
IIx	0	0	0	2
III	0	0	1	0
IV	21	11	6	2
Pneumococcus not isolated	4	1	1	2*
	25	15	12	6

* Staphylococcus pneumonia.

Since no morbidity data are available, it is not certain whether pneumonia is less prevalent in the south than in northern states or equally prevalent but less fatal.

Tables I and II suggest that pneumococcus pneumonia may be as frequent in the isolated rural districts in the south as in the large cities in the north, but is much less fatal.

Age Distribution

In Table III we have summarized the distribution of cases of pneumonia by age groups. The striking feature of Table III is that pneumonia due to Type IV pneumococcus occurred chiefly in children under 15 years of age, whereas pneumonias due to the fixed types of pneumococci were seen chiefly in adults.

TABLE III

Age Distribution of Cases of Pneumonia in Southern Alabama

Age	Total cases	Fixed types of pneumo-cocci	Type IV	Pneumo-coccus not isolated
Under 1 year	1	0	1	0
1– 4 years	9	0	6	3
5–14 "	26	4	19	3
15–44 "	15	4	10	1
45–64 "	4	2	2	0
65 and over	3	0	2	1
	58	10	40	8

Seasonal Distribution of Pneumonia

The community studied was free to a great extent from acute respiratory disease until the first week in January, though there had been mild outbreaks of "colds" in neighboring counties. Daily records were made of maximum and minimum temperature, relative humidity and rainfall. The exact day of onset of each case of pneumonia was recorded. Directly following a week of low temperature, Jan. 1, there occurred a widespread epidemic of colds. Aerobic cultures from the nasopharynx of many individuals during this outbreak showed (a) a great preponderance of pneumococci Type IV, of low virulence to white mice, (b) a large number of influenza bacilli of various types. The time relationship between the cold weather and the prevalence of pneumonia is shown in Chart I. The data seem to

1927 – 1928
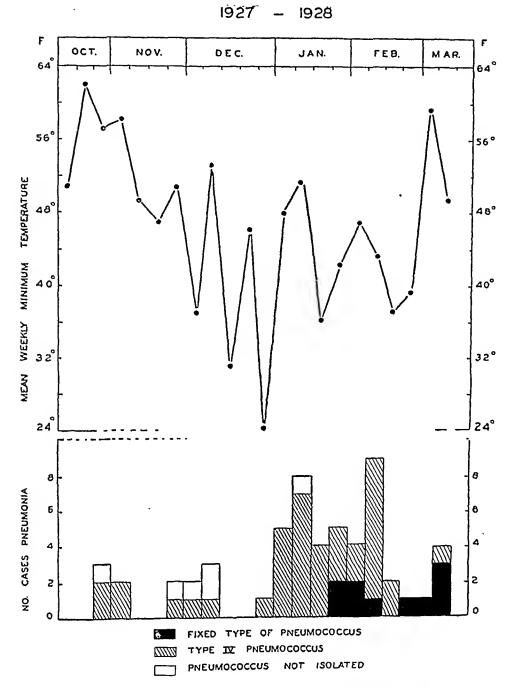
CHART I. Seasonal distribution of pneumonia, Andalusia, Alabama.

indicate that a period of cold weather with attendant suffering and exposure bore a definite relationship to the onset of pneumonia. The sequence was first, cold weather; second, an epidemic of colds; third, pneumonia. The cases of pneumonia did not occur during the coldest weather but 2 to 4 weeks after the weather became warmer.

In individual cases the acute colds developed into pneumonia, usually on the sixth or seventh day of the cold, often following exposure while riding several hours in a wind, or getting rain-soaked and thoroughly chilled when returning from school. The great proportion of the cases occurred in children under 15 years of age and the invading pneumococcus was usually Type IV. Frequently two or more members of the family developed pneumonia at the same time.

TABLE IV

Relationship of Exposure and of Acute Colds to Pneumonia

	Type of pneumococcus		
	Fixed types	Type IV	Pneumococcus not isolated
Sudden onset—no exposure or "acute cold".........	2	4	2
Exposure but no "cold"......................	1	1	0 Total
	3	5	2 10
"Acute cold" antedating pneumonia 5–10 days.....	6	25	3
"Acute cold" plus exposure to chilling rain, etc.......	1	10	3 Total
	7	35	6 48

The relationship of the onset of pneumonia to chilling and exposure and also to acute colds is given in Table IV.

The clinical symptoms in these cases of Type IV pneumonia were typical of lobar pneumonia, but the physical signs were not characteristic of those found in cases of lobar pneumonia due to the fixed and more virulent types of pneumococcus.

The signs of consolidation in the lung were typical but the area involved did not conform to the anatomical outlines of a single lobe. Often areas of consolidation in two different lobes in the same lung were found—sometimes both lungs were affected. The cases could not be called broncho-pneumonia, for small multiple areas of con-

solidation were not found, but rather large areas with typical massive consolidation, sometimes in the axilla but most often in the right base.

The epidemiology of these cases of pneumonia in a sparsely settled rural area is in direct contrast to pneumonia in our larger northern cities where adults are more commonly affected than children and family epidemics are rare; where the attack usually runs a severe course, may or may not be preceded by a cold, and where the physical signs are usually confined sharply to the anatomical outline of a lobe of lung.

It seems a tenable supposition that the isolation, poor roads, lack of frequent contact with one another and with the outside world, have produced in a community of people a low resistance to relatively avirulent pneumococci, whereas under more crowded conditions, there develops a community resistance to avirulent pneumococci, where pneumonia occurs as a rule following an invasion of one of the virulent fixed types of pneumococci.

The mode of life, customs, and habitations of the community studied are closely comparable with those of our forefathers one hundred years ago or more. It is interesting to note that the medical literature of 1810 to 1840 is full of references to epidemic pneumonia.

Warner (1), 1814, describes the epidemic prevailing in New England and New York State. He states "the disease is characterized by an initial chill and prostration and seems related to exposure to wet or cold." Cartwright (2) describes an epidemic in Natchez in 1826 and notes that atmospheric vicissitudes, exposure to inclement weather and intemperance predispose to the disease. MacBride (3), 1813, in describing an epidemic in St. John's Parish, South Carolina, states that most of the deaths occurred in field negroes, but the disease was also seen in whites of the lower classes. When an individual in a family was attacked, nearly all other members developed the disease. McCall (4), 1823, describes a family epidemic in Kentucky; Williamson (5) described an epidemic in 1813 in North Carolina and felt that cold and rain predisposed to the disease. Smith (6), Stearns (7), Mott (8), Mann (9), Eights (10), LeComte (11) and many others describe epidemics of lobar pneumonia in various parts of the eastern United States in the early part of the last century.

It seems probable that the pneumonia in the isolated rural community studied is comparable to the type of pneumonia of pioneer days in the United States, and that the increase of population and the

industrialization of the northern states, producing crowded conditions and frequent contacts, have resulted in the development of a population largely immune to avirulent strains of pneumococci, but responding to certain of the fixed virulent strains.

Family Epidemics of Pneumonia

In seven different families two or more members of the family were ill with pneumonia at the same time. This is contrary to the usual finding in the northern United States, where family epidemics of pneumonia are rare.

The intimate relationship of one case of pneumonia with another suggested the possibility of transmission of the disease from one member of a family to another by immediate contact. If this were the case, then one would expect to find that the various contacts with a case of pneumonia would harbor in their nasopharynx the type of pneumococcus corresponding to that found in the sputum of the patient. To determine this point, a study was made of the nasopharyngeal flora of 26 families in which one or more cases of pneumonia had developed.

Technique

Practically every case of lobar pneumonia which occurred in the area during the winter was seen in consultation with the family physician[2] as soon as the diagnosis of pneumonia was made (usually the second day of the disease). Cultures of the nasopharynx of all contacts were made at once, using the West tube. They were placed directly in blood broth, kept warm during transportation, and plated on 3% horse blood hormone agar plates as soon as possible. The plates were searched after 24 to 36 hours, and typical colonies were isolated and the organism identified. A total of seventy-three contacts were cultured, each of whom had been in close and frequent communication with the pneumonia patient. A summary of the findings is given in Table V.

It might be assumed from Table V that the thirty-two contacts with cases of Type IV pneumococcus who harbored type IV strains in their nasopharynx were infected by actual contact with the patient who was ill with pneumonia in the household. If this were the case, contacts with Type I and Type II pneumonias should harbor corresponding strains. This occurred in only five instances, whereas ten of

[2] There are about twenty-five general practitioners in the area.

the seventeen contacts with Type I pneumonia harbored not Type I but Type IV pneumococcus. It seemed probable, therefore, that some other factor than contact with a case of pneumonia was responsible for the prevalence of pneumococci in the nasopharynx of these individuals.

TABLE V

Prevalence of the Pneumococcus in the Nasopharynx of Individuals in Direct Contact with Pneumonia Patients

	Type of pneumonia in patients to whom contacts were exposed		
	Type I	Type II	Type IV
Contacts harboring pneumococcus:			
Type I......................................	2*	0	0
Type II......................................	0	3	0
Type III......................................	0	1	0
Type IV......................................	10	1	32
No pneumococci found.....................	5	2	17

* Both these contacts developed Type I pneumonia within 3 days after their throat cultures were taken.

TABLE VI

Correlation of the Prevalence of Pneumococci in the Nasopharynx with an Acute Respiratory Infection

Type of pneumococcus found	Acute cold when examined	Just recovered from cold	No history of recent cold
I	2*	0	0
II	1†	0	2†
III	0	0	1†
IV	25	15	3
No pneumococcus found	6	13	5
Total..................	34	28	11

* Contact with Type I pneumonia.
† Contact with Type II pneumonia.

We have noted that practically all cases of pneumonia were preceded by an acute upper respiratory infection and that these colds occurred as family epidemics affecting all members. It seemed possible that these epidemic colds might be associated with an in-

creased prevalence of pneumococci. An analysis was made, therefore, to determine this point. See Table VI.

Table VI shows clearly that a large proportion of the individuals in this study who were harboring pneumococci had colds at the time or had just recovered from them. This is particularly true of the group of individuals who made up the families in which there was a case of Type IV pneumonia. Furthermore, the relative number of pneumococcus colonies on the blood agar plate cultures of the nasophaynx seemed to bear some relationship to the course of the cold. See Table VII.

Tables VI and VII give a very definite indication as to what occurred in these epidemics of colds and the relationship of the cold to pneu-

TABLE VII

Group of Contacts with Type IV Pneumonia; Table Showing Relative Prevalence of Pneumococci Type IV in Acute "Colds"

Proportion of pneumococcus colonies on the blood agar plate in relation to all other colonies	Number of cases			
	1st 3 days of cold	4th to 8th day	1st week after recovery	2nd week after recovery
50 to 100 per cent........................	1	5	3	0
25 to 50 per cent........................	0	3	5	1
Few (less than 25 per cent)................	5	3	1	2
No pneumococci........................	2	3	3	7

monia. Within the first few days of the cold, pneumococci Type IV are found in the nasopharynx of those affected. From the fourth to the eighth day, the pneumococci have so increased as to outnumber all other organisms. As the patient recovers from the cold, the pneumococci disappear and the normal flora of the throat reappear, though pneumococci are frequently found for 10 days to 2 weeks after symptoms of cold have disappeared.

It seems probable that the contacts with cases of pneumonia, particularly the Type IV group, were not infected by their direct contact with a pneumonia patient. The more probable history is as follows: An epidemic of acute colds occurred in a family, usually affecting all members of the family. Coincident with the cold, pneumococci occurred in the nasopharynx in large numbers. In the great majority

of the colds, nasopharyngeal symptoms disappeared after 7 to 1 days, and gradually the pneumococci disappeared also. A sma proportion of the individuals who had an infection with pneumoco of the upper respiratory tract developed pneumonia on the fourth t eighth day of their cold. The attack often followed an exposure o chilling of the body surface, with a resultant extension of the infectio from the upper to the lower respiratory tract. The pneumonia therefore, was an incident in the course of an epidemic of colds.

If this is true, family epidemics of colds associated with pneu mococci must have occurred in which no case of pneumonia developed Such indeed was found to be the case. A complete epidemic of acut colds was studied in a small rural school—cultures being taken befor the colds began, during the epidemic, and after the epidemic hac ceased. A report of this epidemic, together with other similar studies will be made in a subsequent communication.

The assumption might be made that the incitant of these colds is the pneumococcus—especially since there is some contributory evidence in addition to that already presented. One bit of evidence is that these colds were associated with a high leucocytosis—a white blood count of twelve to fifteen or even eighteen thousand being commonly found. Furthermore, one of the present investigators developed a clear-cut nasopharyngeal infection due to pneumococci which was acquired in the laboratory while working with the pneumococcus cultures and which ran a course similar to the family epidemics of colds. The appearance and disappearance of symptoms in this infection were closely correlated with the appearance and disappearance of pneumococci in the nasopharynx.

In one instance, a pure culture of pneumococci Type I was found in the nasopharynx on the second day of a cold in a person who was nursing a patient with Type I pneumonia. 3 days later the nurse developed pneumonia Type I. In two other instances mothers who were caring for children with pneumonia Type IV developed typical colds, and almost pure cultures of pneumococci Type IV were isolated from the nasopharynx on the first or second day of the cold.

But there is evidence also that pneumococci were merely contributory causes and perhaps did not initiate the colds at all. It will be noted from Table VII that pneumococci were not abundant in the

nasopharynx during the first 2 or 3 days of the cold, but were most prevalent from the fourth to the eighth day. This observation suggests that the pneumococci may be secondary invaders and that the cold was initiated by some unknown factor.

Another interesting observation in connection with these family epidemics of colds associated with pneumococci is that Pfeiffer bacilli were frequently found in abundance in association with the pneumocci. In general these organisms appeared later in the cold than the pneumococcus, and were present for a longer period after symptoms had disappeared. No constant type of Pfeiffer bacilli was encountered. Both hemolytic and non-hemolytic forms were found—

TABLE VIII

Table of Correlation between Incidence of "Colds" and Prevalence of Pneumococci and Pfeiffer Bacilli in the Nasopharynx

	Number of cases			
	Acute cold at present	Just recovered from cold	No history of recent cold	Total
Pfeiffer bacilli and pneumococci............	11	6	0	17
Pfeiffer bacilli only......................	4	8	1	13
Pneumococci only........	15	11	5	31
Neither pneumococci nor Pfeiffer bacilli..	5	3	6	14

some requiring V + X factors for growth, others requiring only V factor. Some produced indol—others did not. There was some consistence in the type found in members of the same family but not in the group as a whole. A brief summary of these findings is given in Table VIII.

Table VIII indicates that Pfeiffer bacilli were frequently associated with pneumococci in the acute colds which occurred in the group of contacts with cases of pneumonia.

CONCLUSIONS

1. Pneumococcus Type IV of low virulence was the prevailing organism in fifty-eight cases of pneumonia studied in southern Alabama. Fixed types of pneumococci were not common.

2. Pneumonia was more prevalent in children from 5 to 15 years of age than in adults. As a rule, the disease ran a mild course.

3. Most of the cases of pneumonia gave a definite history of an acute cold antedating the attack of pneumonia by a period of 5 to 8 days. Exposure alone did not seem to predispose to pneumonia, but those with an acute cold who were exposed to chilling of the body surface frequently developed pneumonia.

4. There were seven "family epidemics" of pneumonia. In each instance there was a family epidemic of colds antedating the pneumonia. The pneumococcus was found in large numbers in the nasopharynx of those suffering from colds as well as in the pneumonia patients.

5. The epidemiology of pneumonia in the pioneer days of American history has many points in common with the epidemiology of pneumonia in a rural isolated area in southern Alabama today. This suggests that the crowded conditions and frequent contacts of modern city life have built up a community resistance to avirulent strains of pneumococci.

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STUDIES ON THE DISSOCIATION OF THE HOG CHOLERA BACILLUS

I. The Isolation and Differentiation of Dissociants

By C. P. LI, M.D.

(From the Division of Bacteriology, Department of Pathology, Peking Union Medical College, Peiping, China)

Plate 12
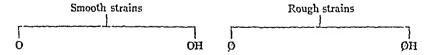
The question whether the dissociation occurring in the paratyphoid group of bacteria follows the H and O type of dissociation of Weil and Felix, the S and R type of Arkwright or a combination of both is still unsettled.

According to Hadley (1) (p. 141) the somatic and flagellar antigen hypothesis of Malvoz should be abandoned since certain nonflagellated bacteria split into 2 forms, R and S, which give the same order of serological reactions as those observed in the R and S types of motile bacteria.

Arkwright and Goyle (2) claimed that the O and H antigens were essentially those of smoothness and roughness. This view was however criticized by White (3) who has represented the variation of the paratyphoid group in a scheme which in a somewhat simplified form is as follows:

O = smooth, heat stable, granular antigen complex.
H = heat labile, floccular antigen complex.
Ø = rough, heat stable. granular antigen complex.

He has also pointed out that the flagellar and somatic agglutinins were identical with the H and O antigens, and that motility was entirely restricted to bacilli giving definite H reactions. No members of the O variety were motile. There are theoretically, therefore, disregarding the specific and nonspecific dissociation of Andrewes (4), and aside from the normal motile smooth (OH) form, three possible forms of variants: the nonmotile smooth (O), the motile rough (ØH), and the nonmotile rough (Ø). Arkwright (5) has demonstrated the existence of these variant forms of *B. paratyphosus* A.

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It is obvious that the dissociation phenomenon of the motile paratyphoid group of organisms is more complex than that of nonflagellated forms like *B. dysenteriae*. The simple S and R conception of dissociation does not seem to be adequate to account for the several variant forms encountered among the flagellated bacteria.

Smith and Reagh (6) first reported the existence of both motile and nonmotile strains in hog cholera bacillus and demonstrated the difference between flagellar and somatic agglutinins. Their work was soon confirmed by Beyer and Reagh (7), Orcutt (8) and others.

In the present investigation observations on the dissociation of the hog cholera bacillus were made by using a special semi-solid medium. Three distinct variant forms arising from the original "normal" strain were observed.

EXPERIMENTAL

Hog cholera bacillus XVI obtained from The Rockefeller Institute, Princeton, N. J., was used. The bacteria derived from the characteristic smooth colonies were actively motile and showed the normal biochemical and serological reactions for this type. Single colonies of the motile smooth (MS) form were selected from 3 successive platings to obtain a pure line culture. This culture was then inoculated in meat infusion broth, incubated at 37°C. for several weeks, and plated in semi-solid medium for study.

The semi-solid medium described by Hiss (9) was used. This medium was found to be useful not only for the isolation of the usual motile and nonmotile forms but also for the differentiation of other variants. The medium contains agar 10 gm., gelatine 160 gm. and meat infusion bouillon 2000 cc. The agar was dissolved in 600 cc. of distilled water by boiling for 20 to 30 minutes. Bouillon was added and the boiling continued for about 10 minutes, during which time, the gelatine was slowly added. Water was added to make up the volume lost by evaporation. The mixture was adjusted to pH 7.4, sterilized in the autoclave for 20 minutes, cleared by sedimentation in an inspissator, and filtered through absorbent cotton at 45°C. The medium was then tubed while hot into large test tubes each containing 15 cc.

This semi-solid medium was inoculated with 0.2 cc. of a suspension of hog cholera bacilli made by adding one loopful of the old broth culture to 30 cc. of normal saline solution, and plates were poured. The medium formed a layer approximately 5 mm. thick. The plates were incubated at 37°C. for 15 to 18 hours and were placed in the refrigerator for about an hour to congeal the medium. They were then examined by holding them above and in front of a Leitz microscope lamp or under the low power lens of a microscope.

Dissociation Forms Encountered

Thus far in the study of colony variation of the typhoid-paratyphoid group, the surface colonies on agar plates have been observed and little attention has been paid to the deep colonies growing in semi-solid medium. In the present study, after the broth culture had been kept at 37°C. for about 3 weeks and plated, only the deep colonies which grew in the semi-solid medium were studied. The following forms of deep colonies were observed:

1. Homogeneous Colonies (Motile Smooth).—These were large, round, disk-like, transparent colonies with smooth margins and surfaces,— the "normal" type form (Fig. 1). The center was as a rule more dense than the periphery, and occasionally appeared as an ill-defined spot. Concentric zones of varying thickness which appeared to be the zones of migration were usually present. Colonies 18 hours old generally measured from 2 to 3 cm. in diameter.

The individual organisms of this form were well-separated and actively motile. They grew in a homogeneous cloud in broth. The growth from an agar slant formed a diffuse suspension when placed in normal saline solution. When grown on the surface of an agar plate, these bacteria formed smooth, round colonies.

This form was designated MS (motile smooth).

2. Irregular Colonies (Motile Rough).—These forms were irregularly shaped, and consisted of clumps or clusters of small secondary colonies (Fig. 2). The characteristics were best observed under low power magnification. Some of the colonies were more or less disk-shaped but the ill-defined margins and surfaces and the presence of clumps of secondary colonies differentiated them from the homogeneous (MS) type. These colonies were much smaller than the homogeneous form, generally measuring from 1 to 3 mm. in diameter.

The bacteria from this form of colony occasionally formed short chains and clumped together. Some of the clumps moved sluggishly as units. Actively motile, individual bacilli were also present. In broth cultures the growth clumped, settled to the bottom and left a clear supernatant fluid. Bacteria from an agar slant culture, when suspended in saline, clumped and precipitated in large membranous flakes.

The colonies formed on the surface of agar plates were rough, granular and irregular, and were larger, under these circumstances, than colonies of the MS, or NS forms.

This form of variant was designated as MR (motile rough).

3. Compact Colonies (Nonmotile Smooth).—The compact colonies had the appearance of small dots (Fig. 3). The average diameter of a colony incubated at 37°C. for 24 hours was from 0.2 to 0.4 mm. Under low power magnification the colonies were yellowish in color. The surface of the young colonies was smooth, while that of colonies more than 48 hours old was slightly uneven.

TABLE I

Comparison of the Four Forms of Hog Cholera Bacillus

	MS	MR	NS	NR
Deep colony in semi-solid medium	homogeneous 2–3 cm.	irregular 1–3 mm.	compact 0.2–0.4 mm.	compact 0.2–0.4 mm.
Surface colonies on agar plates	smooth	rough	smooth	rough
Cell distribution in broth culture	homogeneous cloud (diffuse)	flaky sediment (clumped)	homogeneous cloud (diffuse)	flaky sediment (clumped)
Motility	motile	motile	nonmotile	nonmotile
Precipitation in NaCl and MgCl₂ solution	diffuse	clumped	diffuse	clumped
Resistance to normal rabbit serum	not killed	killed	not killed	killed

The bacilli of this form did not clump and were nonmotile. Like the MS bacilli, these produced a homogeneous, cloudy growth in broth and the growth from an agar slant could be easily suspended in normal saline solution. On the surface of an agar plate the colonies were round and smooth, and were indistinguishable from those of the MS type.

This form of variant was designated as NS (nonmotile smooth).

4. Compact Colonies (Nonmotile Rough).—When bacilli from the NS form of colony were cultivated in broth at 37°C. for several weeks and again plated in semi-solid medium, the same type of compact colony appeared. After this procedure, however, when the bacilli

were transferred to broth the growth in some of the tubes was different.   Instead of the usual homogeneous, cloudy, growth clumps formed which precipitated.   The bacteria from these clumps were nonmotile. In the semi-solid medium compact colonies were formed which were indistinguishable from those of the NS form.   When an agar slant culture of these organisms was suspended in normal saline solution, the growth precipitated in large flakes.   Surface colonies on agar plates were rough, granular and irregular, and were indistinguishable from the MR form.

TABLE II

Reaction of the Four Forms of Hog Cholera Bacillus in Magnesium Chloride Solution

Culture	Time, hrs.	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	H₂O
MS	2	—	—	—	—	—	—	—	—	—	—
	24	—	—	—	—	—	—	—	—	—	—
MR	2	—	+	+	+++	+++	+++	+++	—	—	—
	24	+	+++	+++	+++	+++	+++	+++	±	—	—
NS	2	—	—	—	—	—	—	—	—	—	—
	24	—	—	—	—	—	—	—	—	—	—
NR	2	++	++	+++	+++	+++	+++	+++	+++	—	—
	24	+++	+++	+++	+++	+++	+++	+++	+++	—	—

This form was designated as NR (nonmotile rough).

The characteristics of the 4 forms described above are tabulated in Table I.

In plating the old broth culture as described above, the homogeneous (normal MS) colonies predominated.   The irregular and compact colonies comprised roughly speaking, 1 or 2 per cent of the total.

Further Differential Tests

Salt Sensitiveness.—Arkwright (10) demonstrated that R forms of *B. typhosus* and *B. dysenteriae* agglutinated spontaneously in 0.85 per cent saline solution while the S types did not.   This test was used to distinguish the R from the S bacteria.   In the present work, the test was performed as follows:

Solutions of magnesium chloride and of sodium chloride were used as shown in Tables II and III. Bacteria from 24 hour agar slant cultures of each of the 4 forms were suspended in distilled water. This suspension was added to the various dilutions of the series of the 2 salt solutions and incubated at 50°C. for 2 hours. The reactions were observed at the end of 2 hours and again after the tubes had remained in the refrigerator for 24 hours.

TABLE III

Reaction of the Four Forms of Hog Cholera Bacillus in Sodium Chloride Solution

Culture	Time, hrs.	M/1	M/2	M/4	M/8	M/16	H₂O
MS	2	—	—	—	—	—	—
	24	—	—	—	—	—	—
MR	2	+	+	—	—	—	—
	24	+	++	++	++	—	—
NS	2	—	—	—	—	—	—
	24	—	—	—	—	—	—
NR	2	+	+	+	+	—	—
	24	+++	+++	+++	+++	—	—

— = no precipitation.
\+ = moderate precipitation.
++ = marked precipitation.
+++ = complete precipitation, supernatant fluid clear.

The difference in behavior of the 4 forms under these conditions are shown in Tables II and III. The MR and NR forms were clumped by both magnesium chloride and sodium chloride while the MS and NS forms were not. The magnesium chloride was more effective than the sodium chloride.

Survival in Normal Rabbit Serum.—Since the R forms of certain other bacteria are more subject to phagocytosis and destruction than the S forms, attempts were made to test the difference in resistance of the 4 forms of hog cholera bacillus to the bactericidal action of normal rabbit serum.

Broth cultures of the 4 forms of hog cholera bacilli were diluted

1:1,000,000 in saline.   One part of each suspension was mixed with five parts of normal rabbit serum in small tubes.   A control set of the bacterial suspension was mixed in normal saline solution.   The tubes were incubated at 37°C. for an hour after which 0.2 cc. from each tube was plated.   The number of colonies on each plate was counted after 24 hours incubation.

Organisms of the MS and NS forms were resistant to the serum and many colonies appeared on the plate, but only a few of the bacteria of the MR and NR forms survived the effects of the serum, and far fewer colonies appeared when these forms were plated.   The results are shown in Table IV.

TABLE IV

Comparative Resistance of the Four Forms of Hog Cholera Bacillus to the Bactericidal Action of Normal Rabbit Serum

Culture suspended 1 hour at 37°C. in	Number of colonies on agar plates after 24 hours incubation			
	MS	MR	NS	NR
Rabbit serum..............................	299	2	353	87
Saline....................................	223	202	292	215

Resistance to Carbolic Acid.—The difference in resistance of the 4 forms of hog cholera bacilli to the bactericidal action of phenol was not decisive.   Bacilli of the smooth, MS or NS forms were, however, somewhat more resistant than those of the rough, MR or NR forms.

Other tests when applied to the 4 forms failed to show any striking differences.   Bacilli from all 4 forms were Gram-negative, short rods. All forms fermented dextrose, mannite, maltose and xylose and produced acid and gas.   Lactose, sucrose and arabinose were not fermented.   The action on dulcite was irregular, differences in the rate of fermentation appearing even in a series of tubes inoculated with bacilli derived from a single colony.   Indol and hydrogen sulphide were not produced by any of the bacilli.

DISCUSSION

For the differentiation of the original form of hog cholera bacillus and its 3 variant forms both semi-solid and solid agar media were

employed.   It is obvious that the examination of the colonies produced by each of the 4 forms of bacilli on the surface of agar plates alone, serves only to distinguish broadly the smooth from the rough strains. The dry surface of the agar inhibits migration of the motile bacilli and prevents contrast between the motile and nonmotile forms.   For this reason, among the surface colonies on agar, it was only possible to differentiate the smooth (MS, NS) forms from the rough (MR, NR) forms.   Even under these conditions the differences between the S and R forms were often not decisive.   Further microscopic tests are necessary to determine motility.   No differences were detected macroscopically between the MR and the NR colonies on the one hand and between the MS and NS colonies on the other.

When colonies of the 4 forms grow under the surface in semi-solid media motile and nonmotile characteristics appear.   The semi-solid medium permits migration of the motile bacilli which form large diffuse colonies while the nonmotile bacilli form minute compact colonies. Thus it follows that the motile smooth (MS) colonies, which on agar surfaces appeared like the nonmotile smooth (NS) colonies, now become many times larger than the NS colonies due to the centrifugal migration of the motile (MS) bacilli.   At the same time the semi-solid medium permits spontaneous agglutination of the motile rough (MR) bacilli, which form clusters of secondary colonies thus adding another macroscopic differential feature.   Differentiation between the 2 forms of small, compact colonies, nonmotile smooth (NS) and nonmotile rough (NR), is not as clear in the semi-solid media as on agar surfaces since neither form is motile.   On agar plates the smooth and the rough surfaces of the colonies serve to differentiate the NS from the NR form.   Therefore, for a complete differentiation of the 4 forms several tests are desirable: growth on agar plates, growth in semi-solid media, and microscopic tests or growth in broth to determine clumping of the rough forms.

SUMMARY

When the "normal" MS form of hog cholera bacillus was grown for 3 weeks in broth and subsequently plated in semi-solid media, 2 variant forms appeared, a motile rough (MR) form and a nonmotile

smooth (NS) form.   The NS form subsequently gave rise to a third variant, namely, the nonmotile rough (NR) form.

It is evident from the observations reported that in the case of the hog cholera bacillus the simple S and R conception of microbic dissociation is inadequate to account for the several variant forms.   It appears necessary to combine the S and R with the H and O or the flagellar and somatic antigen conception to account for the phenomenon.

The dissociation is represented by the following diagram:

$$\begin{array}{ccc} & \text{MS (normal)} & \\ \swarrow & & \searrow \\ \text{MR} & & \text{NS} \\ & & \downarrow \\ & & \text{NR} \end{array}$$
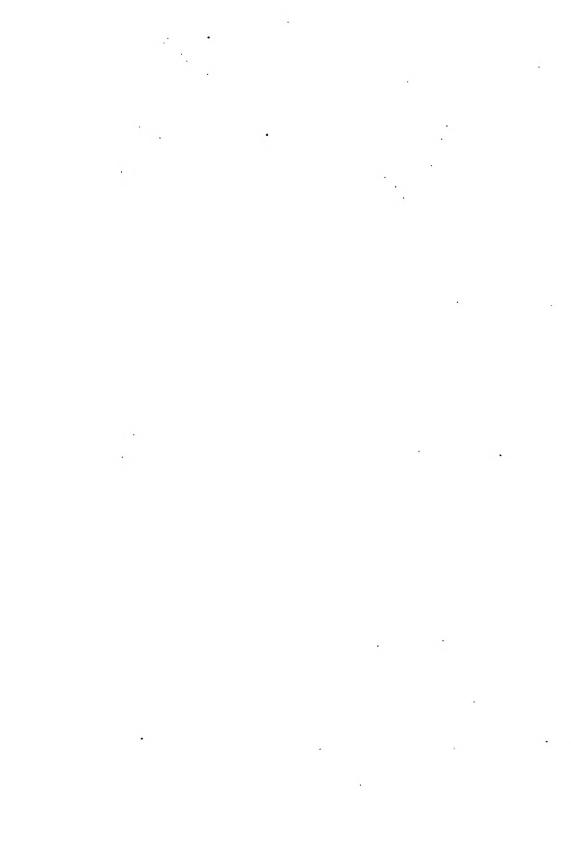
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EXPLANATION OF PLATE 12

FIG. 1. Normal MS colonies of hog cholera bacilli 17 hours old, growing in semi-solid medium.   Natural size.

FIG. 2. MR colony 17 hours old in semi-solid medium, showing the secondary colony formation.   X 30.

FIG. 3. NS colony 17 hours old in semi-solid medium.   NS and NR colonies appear alike under these conditions.   X 30.

STUDIES ON THE DISSOCIATION OF THE HOG CHOLERA BACILLUS

II. Serological Reactions, Virulence and Stability of the Variant Forms

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In a previous paper (1) a study of the dissociation of the hog cholera bacillus was presented. By using both semi-solid and solid agar media the appearance of 3 distinct variant forms was observed arising from the "normal" motile smooth (MS) strain. The 3 variant forms which appeared were designated motile rough (MR), nonmotile smooth (NS), and nonmotile rough (NR). Briefly the appearance of the colonies of the 4 forms which grew deeply in the semi-solid medium was as follows: (a) MS,—large homogenous colonies, (b) MR,—smaller irregular colonies, (c) NS,—minute compact colonies and (d) NR,—minute compact colonies. To differentiate the latter 2 forms macroscopically, it was necessary to observe the colonies as they grew on the surface of agar plates. Under this condition, the smooth surfaces of the NS colonies were distinguished from the rough surfaces of the NR colonies.

The bacteria from the S colonies, both motile (MS) and nonmotile (NS), grew diffusely when cultivated in broth and remained in diffuse suspension when placed in saline solutions. Bacteria from the R colonies, both motile (MR) and nonmotile (NR), grew in clumps in broth media and when suspended in salt solutions clumped and precipitated in flakes. It was also observed that bacilli from both S forms of colonies were more resistant to the bactericidal action of phenol and of normal rabbit serum than the organisms derived from the R colonies.

In the present report further differential tests were applied to

the 4 forms of the hog cholera bacillus.   A study of the serological reactions, of the virulence and of the stability of the 4 forms was made.

Serological Reactions

Reference was previously made (1) to a schematic diagram by White (2) representing the dissociant forms of the paratyphoid group of bacteria.   The probability that the flagellar and somatic antigens were identical with the H and O antigens respectively was

TABLE I

Theoretical Agglutination of the Variant Forms of Hog Cholera Bacilli Assuming that $MS = OH$, $MR = \emptyset H$, $NS = O$ and $NR = \emptyset$

Antigen	Immune sera			
	Anti-MS (OH)	Anti-MR ($\emptyset$H)	Anti-NS (O)	Anti-NR ($\emptyset$)
MS (OH)	+	+	+	—
MR ($\emptyset$H)	+	+	—	+
NS (O)	+	—	+	—
NR ($\emptyset$)	—	+	—	+

+ = agglutination.

— = no agglutination.

O = smooth, heat stable, granular, somatic antigen complex.

H = heat labile, floccular, flagellar antigen complex.

$\emptyset$ = rough, heat stable, granular, somatic antigen complex.

also remarked.   Under this assumption the 4 forms of hog cholera bacillus, MS, MR, NS and NR may be represented by the symbols OH, $\emptyset$H, O and $\emptyset$ respectively ($\emptyset$ = the rough form of O). Accordingly, the theoretical serological reactions of the various forms are shown in Table I.   A study of the actual serological reactions was then made.

Methods

Preparation of Sera.—For the preparation of the immune sera, bacteria from agar slant cultures were suspended in salt solution and killed at 56°C.   Rabbits were then immunized by injecting suspensions of the 4 forms of bacilli intravenously 6 or 7 times at 5 to 7 days intervals.   A week after the last injection the animals were bled to death and the serum was obtained.

Sera were absorbed with heavy suspensions of living bacteria in 0.1 per cent salt solution (stronger solution of salt caused spontaneous clumping of the R forms and was not used). The mixtures were incubated at 37°C. for 2 hours, placed in the ice box for 24 hours and finally centrifuged to regain the diluted serum. The final dilution of the sera was 1:20.

Agglutination Tests.—The bacteria to be tested were derived from agar slant cultures and were suspended in 0.1 per cent salt solution. Dilutions of sera in 0.1 per cent salt solution, varying from 1:40 to 1:10240, were made to which the bacterial suspensions were added. Suspensions of bacteria in 0.1 per cent salt

TABLE II

Agglutination Reactions Actually Observed

Culture tested	Agglutination titer			
	Anti-MS serum	Anti-MR serum	Anti-NS serum	Anti-NR serum
MS	5120 F	5120 F	640 G	~
MR	5120 F	5120 F	160 ?	160 G
NS	640 G	~	640 G	~
NR	~	160 G	~	160 G

F = floccular.

G = granular.

~ = no agglutination.

? = differing from theoretical result.

solution served as control tests. The tubes were then incubated at 50°C. for 2 hours. Readings were made at the end of this time and again after the tubes had been in the ice box for 24 hours.

The results of the agglutination tests are shown in Table II. The figures represent the highest dilution in which macroscopic agglutination occurred. The letter F indicates a floccular type of precipitation and G indicates granular precipitation.

It is evident that the actual agglutinations represented in Table II correspond closely to the theoretical reactions predicted in Table I. An exception is noted by the agglutination of MR bacteria in a relatively low dilution of anti-NS serum. The reason for this is not clear and is at present unexplained. It is possible that the anti-NS serum contained some H agglutinins which was indicated by the fact that after the MR bacilli were heated to 100°C. for

30 minutes to destroy the H antigen, no agglutination in anti-NS serum occurred.

The titer of the anti-NR serum was low and attempts to produce a stronger anti-serum were not successful.

Different types of agglutination were observed as indicated in the table. Bacteria of the MS and MR forms when agglutinated by either anti-MS or anti-MR sera promptly formed large, loose, floccular clumps, due to the presence of the H antigen and antibody. MS bacilli in anti-NS serum and MR bacilli in anti-NR serum

TABLE III

Results of Absorption Tests

Bacterial suspensions	Anti-MS (OH) serum			Anti-MR (ØH) serum			Anti-NS (O) serum			Anti-NR (Ø) serum	
	Unabsorbed	Absorbed with MR bacilli	Absorbed with NS bacilli	Unabsorbed	Absorbed with MS bacilli	Absorbed with NR bacilli	Unabsorbed	Absorbed with MS bacilli	Absorbed with MR bacilli	Unabsorbed	Absorbed with MR bacilli
MS (OH)	5120 F	640 G	2560 F	5120 F	—	5120 F	640 G	—	640 G		
MR (ØH)	5120 F	—	2560 F	5120 F	160 G	5120 F	160 ?	—	—	160 G	—
NS (O)	640 G	640 G	—				640 G	—	640 G		
NR (Ø)				160 G	160 G	—				160 G	—

The figures represent the highest dilution in which macroscopic agglutination occurred.

F = floccular agglutination.

G = granular agglutination.

? = differing from theoretical result.

slowly formed into hard, small, granule-like clumps. Bacteria of the NS and NR forms formed similar small granular clumps whenever agglutinated by any of the 4 anti-sera, due to the presence of the O or Ø antigen and O or Ø antibody.

Absorption Tests.—The results of absorption tests which are given in Table III confirmed the results obtained by the ordinary agglutination reaction. The floccular H agglutinin was absorbed from the anti-MS (OH) serum by the MR (ØH) bacilli, but the granular (O) agglutinin remained intact. MR (ØH) bacilli absorbed the

granular (Ø) antigen from the anti-NR (Ø) serum.   Anti-NS (O) serum was unaffected by MR (ØH) bacilli.

Similarly the MS (OH) bacteria exhausted the floccular (H) agglutinin from the anti-MR (ØH) serum as well as the granular (O) agglutinin from the anti-NS (O) serum, since these bacilli possess both O and H antigens.   The granular (Ø) agglutinin in the anti-MR (ØH) serum was, of course, unaffected.

Lastly NS (O) bacteria had no influence on the H agglutinin in anti-MS (OH) serum and the NR (Ø) organisms absorbed the

TABLE IV

Virulence of the Four Forms of Hog Cholera Bacilli for Hamsters (Chinese Field Mice)

Culture tested	Dose, cc.	No. used	No. died	Mortality
MS	0.1	5	5	100%
	0.001	10	10	100%
	0.000001	5	4	80%
MR	0.1	5	0	0
	0.1	10	0	0
NS	0.1	5	5	100%
	0.001	10	5	50%
NR	0.1	5	0 (abscess)	0
	0.1	10	0 (abscess)	0

Animals were observed for 30 days.

granular (Ø) agglutinins from the anti-MR (ØH) serum but left its H agglutinin intact.

It should be mentioned that for the various serological tests only typically reacting bacteria were used.   Indefinite, intermediate, or confusing forms which reacted atypically were frequently encountered and were discarded.

Virulence

Besides the differences in the serological reactions of the 4 forms of the hog cholera bacillus, striking differences in virulence for hamsters (Chinese field mice) and rabbits were observed.   Varying

amounts of 24 hour old broth cultures of the 4 forms of bacilli were injected subcutaneously into hamsters. The results of the experiment are given in Table IV.

From the table it appears that in general, as in other bacterial species, the S forms were virulent and the R forms were not. The virulence of the MS form seems to be somewhat greater than that of the NS form. The NR form, however, can probably not be considered to be entirely avirulent. Hamsters receiving 0.1 cc. doses of the NR form subcutaneously occasionally died and abscesses usually developed at the site of inoculation in those that survived. The MR form on the other hand produced no gross pathological changes. Both the MR and the NR forms were frequently virulent for hamsters when injected in large doses (0.5 cc.) intraperitoneally.

Rabbits, when inoculated with the 4 forms of bacilli, reacted in a similar manner. The MS form caused death in 7 days after the subcutaneous inoculation of 0.000001 cc. of broth culture. Of 2 rabbits inoculated with 0.1 cc. of an NS culture, one died after 9 days and the other recovered after a period of fever lasting about 3 weeks. Both NR and MR organisms failed to kill rabbits even in 0.1 cc. doses, but the animals receiving NR bacilli, as in the preceding experiment, developed fever (40°C.) and appeared ill for several days but recovered.

Cultures were made from the spleen of all animals which succumbed during the course of the virulence tests. The same forms of variant bacilli as those used for inoculation were recovered.

Studies on the Stability or Reversibility of the Variant Forms

Reversion in Broth.—Attempts were made to induce reversion from one form to another. The 3 variant forms were repeatedly grown in meat infusion broth. Transfers were made twice daily for 32 days. At intervals during this procedure the cultures were plated in semi-solid media and on the surface of agar plates, and examined. The bacteria were also tested for motility and for clumping in salt solution.

During the experiment, the NR and the NS forms invariably reproduced only their respective kinds. No reversion occurred. However, after several transfers of the MR form, many MS colonies

of the original "normal" strain appeared among the MR colonies. Certain MR forms therefore reverted to the original MS form.

Effect of Animal Passage.—Broth cultures of the 3 variant forms were injected intraperitoneally into hamsters. Large doses of MR and NR strains were virulent when injected intraperitoneally instead of subcutaneously. The 3 strains were passed through 15 mice by intraperitoneal injection of peritoneal washings from mice killed by the bacilli. At intervals during this procedure the bacilli were plated in semi-solid medium and on solid agar, and the colonies were examined.

The NS and NR forms were unaffected by mouse passage and did not revert to any other form. The MR form, on the other hand, showed a tendency to revert to the MS form. After the 6th passage a few MS colonies appeared among many MR colonies. During subsequent (15) passages the proportion of MS to MR colonies gradually increased.
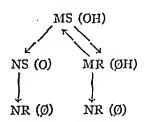
Effect of Growth in Immune Serum.—Since other bacteria have been found to revert from R to the S form during growth in anti-R sera, similar trials were made with hog cholera bacilli. The 3 variant forms NS, MR and NR were transferred every other day in broth containing 10 per cent of NS, MR and NR anti-sera respectively for 2 weeks. The cultures were plated and examined at intervals. The NS form showed further dissociation into the NR form. NR bacilli also appeared among the MR forms during the transfers. The NR forms arising from the MR form were identical with the NR forms which were derived from the NS bacilli. The NR form remained unchanged. It appeared from this test that growth in anti-R sera did not cause a reversion of the NR or MR forms to the original form but did cause further dissociation of the MR and NS organisms.

SUMMARY

The experiments recorded in the present paper confirm the existence of 4 forms of the hog cholera bacillus described in a previous paper (1), namely the "normal" type strain MS and its 3 variants, MR, NS and NR. Serological evidence is also presented to show that the symbols MS, MR, NS and NR represent the similar

conceptions of previous investigators who have used the letters OH, O, ØH and Ø respectively to designate variant forms of other organisms.   It has been shown that the S form of the hog cholera bacillus, as the S forms of other bacteria, is more virulent for laboratory animals than the R forms.

In regard to the reversibility of one form to another it was found that by transfer in broth or by passage through mice the MR form showed a tendency to revert to the original MS form. Transfer in broth and animal passage, however, failed to induce any variations in the NR or NS forms.   Growth in homologous immune sera did not cause reversion to original forms but in fact provoked further dissociation of the MR to the NR form and also of the NS to the NR form.   The MR form may revert to the original normal MS form or may dissociate further into the NR form and is, therefore, the most unstable of the variants.   Further attempts to induce changes in the other variants were not made. The dissociation as observed may be represented thus:

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RELATION OF VACCINAL IMMUNITY TO THE PERSISTENCE OF THE VIRUS IN RABBITS

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In a recent preliminary communication (1) we described briefly the isolation by cataphoresis of vaccine virus from rabbits entirely recovered from infection with it. By the method the virus can be concentrated from suspensions of tissues which fail to reveal infectivity in the usual tests of animal inoculation. We suggested that the immunity in virus diseases may be linked with the persistence in the body of living virus. In the present paper we intend to elaborate the subject and to offer additional experiments to support our conclusions.

The presence of virus in animals recovered from various virus infections. One of the outstanding instances of prolonged retention of a virus after recovery was that described by de Kock (2), in which the blood of a horse proved infectious seven years after an attack of equine pernicious anemia. As concerns foot-and-mouth disease (3), it was reported that a bull was infectious $2\frac{1}{2}$ years after recovery (Bang), and cattle after six months (Loeffler); that the blood of a dead guinea pig contained the virus 198 days after inoculation (Fortner), and that material from the hoof of a healed animal yielded the active agent 34 days after infection (Olitsky, Traum, and Schoening). Lucas and Osgood (4) showed that the virus of poliomyelitis might be present in monkeys for five months after recovery from an acute attack of the experimental disease. They found (5) the incitant of poliomyelitis in the filtered nasal secretion of a child four months after a second attack of the disease. The first attack of the disease had occurred two years previously, and Lucas and Osgood considered the second attack a recurrence, believing that the child had harbored the virus during the interval.

The salivary gland virus of the guinea pig can be obtained at any stage after infection and throughout the subsequent period of immunity (6). A similar condition prevails in contagious epithelioma of fowls (7). And the blood of horses injected with African horsesickness virus is infectious for three months (8). Moreover, Rivers' Virus III has been found in a transplantable neoplasm of the rabbit so long as the tumor lasts. It should be noted that the host meantime developed an immunity to the virus (9).

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Noguchi found vaccine virus in the lymph nodes of a rabbit 28 days after inoculation and 22 days after cutaneous immunity was established (10). Recently, Douglas, Smith, and Price recovered the virus from the ovary or testicle, from the adrenal, tongue, and brain of rabbits 41 days after infection (11). Vaccine virus, like Virus III, can persist in neoplasms of rabbits so long as these exist (12, 13). Rivers and Pearce found it after 64 days, and it had induced a resistance in the rabbit against vaccinal infection (13). Winkler (14) has reviewed the literature on the persistence of vaccinia and small pox viruses in man and cited several significant instances.

Sufficient references have now been given to indicate that the persistence of viruses in recovered, and immune, animals is not an unusual phenomenon. Special mention should be made, however, of the fact that in 1887, L. Pfeiffer concluded from clinical observation mainly that a patient recovered from vaccination, and consequently immune, harbors the virus indefinitely, immunity being lost when it disappears from the body (15).

Cataphoresis of vaccine virus. Douglas and Smith (16) have already shown that vaccine virus as contained in suspensions of tissues is, under ordinary conditions of hydrogen ion concentration, electronegative. The virus is capable of migrating in an electrical field to the positive pole at from $pH = 5.5$ to 8.4. In their experiments, Todd's apparatus (17) for determining the charge of bacteriophage was used.

It should be emphasized that in the present state of knowledge one cannot say definitely whether the virus itself or material containing the active agent carries the electronegative charge.

EXPERIMENTAL

Mode of Procedure

Certain steps in the mode of procedure for the isolation of small quantities of vaccine virus by cataphoresis were taken on empirical grounds. After a few preliminary experiments we discarded rheostats and substituted the resistance offered by the suspensions of tissues themselves. We employed distilled water for making the suspensions, of which about 90 to 96 cc. were used in each test, and we permitted the current to flow over a period of three hours. That the combination of these and other circumstances, to be described immediately in detail, proved satisfactory is exemplified by the fairly regular way in which virus was obtained from a number of samples that were inactive by ordinary tests.

The vaccine virus. The type of vaccine virus which was used was the neuro-vaccine of Levaditi (18), which we found generally active in dilutions of 1:100 million of unfiltered testicular suspensions. Rabbits were injected intracutaneously from 12 to 178 days prior to the cataphoresis test with testicular virus, and all showed, after inoculation, the characteristic vaccinal lesions in the skin. At the time these rabbits were subjected to the test for presence of virus, they were, however, wholly recovered from the infection.

The tissue suspension. Since it was desired to test the state of immunity of the rabbit after obtaining the results of cataphoresis, the skin, testicles, or spleen were removed under ether anesthesia and the animal allowed to recover.[1] The tissues were minced, then ground with sand, and made up with 100 cc. of distilled water to a homogeneous suspension. The water employed had, as a rule, a hydrogen ion concentration of 6.5 to 6.8. In some instances in which it was found desirable to increase the ionic content of the suspension, we employed as a vehicle, not water but phosphate-buffer solution (0.25 per cent KH_2PO_4) at pH = 7.5 or 7.6. The mixture was centrifuged very gently for the purpose of sedimenting large particles. The more or less turbid suspensions were used for cataphoresis, and among the several samples thus obtained, the hydrogen ion concentration varied from 6.3 to 7.8. This wide range did not influence the results of the test apparently. It should be added that the entire procedure was carried out under sterile conditions.

The cataphoresis apparatus. An adaptation was made of the method employed by Todd (17) and by Field and Teague (19). The apparatus had the following arrangement:

A lamp cord was plugged into the house current socket which carried a direct current of from 116 to 120 volts and 10 amperes. At a point on the positive wire of the cord was attached a milliampere meter with gradations on dial permitting single milliampere readings. To both positive and negative terminals zinc electrodes were attached which were cleaned at the beginning of the experiments and between times with 10 per cent sulphuric acid, and then covered with an amalgam of mercury, so as to render them non-polarizable. The electrodes were placed in a glass dish containing 10–20 cc. of supersaturated zinc sulphate solution.

On ring stands were clamped three sterilized, glass U tubes, the over-all measurements of each being 10 cm. height and 5.5 cm. width, with a uniform bore of 1.5 cm., and a capacity of from 40–44 cc. The curved part of each U tube was plugged with sterile cotton so as to prevent convection currents. The three tubes were connected with each other and with the positive and negative electrodes by means of small, glass connecting tubes, also U-shaped. The over-all measurements of the connecting tubes, with the exception of the first, the one which extended from the positive electrode to the first U tube, were 6.5 cm. height, 3 cm. width, the uniform bore being 4 mm. The first connecting tube was constructed with the electrode arm much longer, namely, 12 cm., which prevented the zinc sulphate from wandering into the material in the first U tube. The connecting tubes were filled with agar gel consisting of 1 per cent agar and 1 per cent sodium chloride in distilled water and sterilized. The pH of the agar was 6.6, but positive results could also be obtained when the reaction was adjusted to pH = 7.5 with phosphate-buffer solution or sodium hydroxide. Care was exercised to prevent inclusion of air bubbles in the tubes, which could offer gaps to the current. In addition, a volt meter with single volt readings was employed to obtain the drop in potential through the system. This varied from 115 to 119 volts in the system of U tubes,

[1] All operations on animals were made with the help of complete ether anesthesia.

or 1 to 2 volts less than the house current voltage, and was therefore between about 38 to 39 volts for each.

The test. After removal of about 5 cc. of the tissue suspension intended for cataphoresis, part of which was used to determine the hydrogen ion concentration and part the activity of the suspension, from 30–32 cc. of the remainder were poured into each of the U tubes. The connecting tubes were then adjusted so that each end penetrated the fluid in the U tube to the depth of 1 to 1.5 cm. The U tubes were plugged with sterile cotton and the glass part of the apparatus was covered with sterile cotton or gauze to prevent the entrance of air contaminants. When hydrostatic equilibrium was established, the current was turned on and allowed to run for three hours. After the first 20 minutes there appeared, as a rule, a clearing at the cathodic part of the suspension and a clouding at the anodic part of both the suspension and the agar of the connecting tubes. The change was progressive and reached its maximum after about two hours.

When the current was discontinued after three hours, the connecting tubes were removed. From 2 to 3 cm. of the agar at each end, corresponding to positive and negative poles, was scooped out with a cataract knife. Into one mortar was placed the three anodic portions, and into another the three cathodic specimens. These were ground with 1.5 to 2 cc. of Ringer's solution. The ground agar emulsion was then injected intratesticularly by the usual method into rabbits, the pooled anodic material into one animal and the cathodic into another. If the agar contained virus, characteristic vaccinal orchitis developed, first showing itself in 24 to 48 hours. In any event, after three to four days, the testicles were removed and suspensions of them were injected into the skin of normal and of vaccine-virus immune rabbits, to confirm the original diagnosis. Finally, either before or after it was learned that virus was present or absent, the animal from which the tissue was removed for the cataphoresis test was examined for its immunity. This was done by injecting endermically active vaccine virus in different dilutions of 1:10 to 1:10,000. A control normal rabbit was similarly inoculated for comparison. Immune animals showed within 24 hours the transitory Jenner-von Pirquet "immediate reaction," that is, a hyperemia at the site of the injections of the 1:10 to 1:100 dilutions, whereas the normal rabbits responded at a later period with the characteristic vaccinal lesions and subsequent secondary pustules in the case of all the dilutions.

As preliminary tests, we studied by means of cataphoresis vaccine-virus infected testicles of rabbits, at the height of reaction, suspended in phosphate buffer solution at hydrogen ion concentrations of 6, 7.2, 7.5, and 8. In thoroughly controlled experiments, at all four reactions the virus was recovered only at the positive pole. We thus confirmed the earlier observation of Douglas and Smith (16) and also controlled the mode of procedure which we adopted.

RESULTS

Presence of virus in recovered rabbits. Rabbits were examined which had been injected intracutaneously 12, 51, 56, 114, and 133 days before with neurovaccine virus. The animals had responded to the inoculation with characteristic vaccinal skin lesions. The suspensions of the testicles failed in each case to reveal vaccine virus by the ordinary test of animal inoculation; but, on the other hand, they yielded active virus after cataphoresis. The identity of the virus was revealed, first, by the characteristic dermal and testicular reactions following the injection in the manner described of pooled anodic material into normal rabbits, and secondly, by either the "immediate reaction" of immunity (the Jenner-von Pirquet phenomenon), following the injection of the same material into vaccine-virus recovered animals, or by absence of any reaction in such rabbits. The corresponding substance collected at the cathode was uniformly inactive.

It was found that the active agent could be obtained from the testicles of rabbits 133 days after inoculation and 123 days after complete recovery from cutaneous vaccine-virus infection.

Relation of persistence of virus to immunity. The following protocol illustrates the relation of immunity to the persistence or loss of virus in rabbits which had been inoculated with it a considerable time before the test for immunity was made.

Six rabbits were used in this experiment.[2] Rabbit A had been injected intracutaneously with active neurovaccine virus 133 days before the time of cataphoresis; Rabbit B, 143 days, and Rabbit C, 114 days. In the case of Rabbit D, a portion of the skin was removed for test 121 days after the cutaneous vaccination, a testicle 128 days after it, and the spleen 133 days after. The testicles of Rabbit E were taken after 171 days and the spleen after 178 days; the testicle of Rabbit F after 170 days, and the spleen after 175 days.[1] These animals had been vaccinated as follows:

Rabbit A had been injected endermically with neurovaccine virus in three sites. Into each of two areas was put 0.25 cc. of normal rabbit serum plus 0.25 cc. of virus, and into the third, 0.25 cc. of Ringer's solution and 0.25 cc. of virus. After 48 to 72 hours all the sites showed characteristic vaccinal lesions, but the individual reactions were not marked. Three other rabbits (controls for the activity of the virus) injected in a like manner with the same virus revealed similar changes in the skin.

Rabbit B had been treated as was Rabbit A and it responded in a like manner to the injections.

Rabbit C had been inoculated intracutaneously with a special mixture of virus plus an extract of normal rabbit testicles—a combination which, as Duran-Reynals has shown, enhances the activity of vaccine virus (20). After the endermic inoculation of 0.25 cc. of the virus and 0.5 cc. of the normal testicular extract, a typical lesion was noted which, on the fifth day, had spread considerably over the skin and was surrounded by numerous secondary pustules. The reaction was much more severe than that noted in the other five rabbits.

Rabbits D, E, and F had been injected endermically with usual stock neurovaccine testicular virus and responded to a moderate degree with dermal vaccinal lesions.

Suspensions of the organs were prepared for cataphoresis and the tests were conducted as already described. The following results were obtained:

The testicular suspension derived from Rabbit A 133 days after the endermic inoculation of virus showed after cataphoresis no activity in the testicles of normal rabbits; nor was virus found in the suspension by animal inoculation before applying the cataphoresis test. Rabbit A was then—5 days later—subjected to an immunity test. It was inoculated into the skin at various points with active testicular virus in dilutions of 1:100 to 1:6,400—to all of which the rabbit responded with characteristic primary and secondary vaccinal lesions.

A summary of cataphoresis and immunity tests on the remaining five rabbits is given in the following table.

Rabbit	Cataphoresis of suspension of	Days after endermic inoculation of virus	Virus recovered	Immunity test	
				Days after endermic inoculation of virus	Result
B	Testicles	143	No	158	Not immune
C	Testicles	114	Yes	119	Immune
D	Skin	121	No	133	Immune
	Testicles	128	No		
	Spleen	133	Yes		
E	Testicles	171	No	185	Not immune
	Spleen	178	No		
F	Testicles	170	No	183	Not immune
	Spleen	175	No		

As the protocol and the table show, four of the rabbits which had been inoculated endermically with vaccine virus and responded with characteristic vaccinal lesions failed to yield virus from their tissues

133 to 178 days later. Two rabbits inoculated similarly yielded virus, one from the testicle after 114 days, the other from the spleen after 133 days. The tissue suspensions from these latter animals were ineffective by the usual tests of animal inoculation, but active virus was obtained from them by cataphoresis. The rabbits which failed to yield virus also failed to show immunity, whereas those which retained the virus exhibited a specific resistance to virus infection.

In one instance, the virus could not be obtained from the skin on the 121st day after intracutaneous injection, nor from the testicles on the 128th day, but could be recovered from the spleen on the 133rd day. The single observation suggests that the spleen, as well as the testicle, may be concerned with the retention of virus.

Relation of persistence of virus to inherited immunity. Béclère, Chambon, and Menard (21) give an instance suggesting that vaccinal virus may be transmitted through the placenta and affect the fetus.

A patient was vaccinated during infancy and re-vaccinated on entry into the hospital, when she was eight months advanced in pregnancy. The re-vaccination caused an "immediate reaction" of hyperemia, showing that she still retained immunity. Parturition occurred one month later, when blood serum obtained from both the mother and the new-born child (umbilical cord blood) showed distinct virus-neutralizing power. In addition, two attempts at vaccination of . the infant, at 6 and 14 days' age, were unsuccessful.

Ohtawara (22), in studying this problem by means of experiments on rabbits, also concludes that vaccine virus passes through the placenta and can consequently be found in the young. He believes, however, that the virus is present in the new-born only when it exists in the blood of the mother at the time of parturition.

The next experiment, while providing a parallel to this clinical instance, adds support to the view that there may be a relationship between the persistence of vaccine virus in an animal's body and immunity to it.

We injected two pregnant rabbits endermically with vaccine virus, and later, after the young were born and a few weeks' old, each of the litter was subjected to a test for the presence of virus and of immunity.

a) Rabbit A was inoculated intracutaneously with active virus 16 days before giving birth to three young. The animal responded with characteristic primary

and secondary vaccinal lesions. Two days before the birth of the young, the rabbit was normal in appearance.

Two of the young, both females, were tested 32 and 62 days respectively after birth by the injection endermically of active vaccine virus in dilutions of 1:100 to 1:1000. They proved completely resistant to infection. A male of the litter was castrated under ether when 35 days old and allowed to recover, for the purposes of a later immunity test. The testicular suspension as such proved inactive, but after cataphoresis anodic material induced in a normal rabbit characteristic vaccinal orchitis. The height of the reaction occurred on the fifth day, when the involved testicle was removed and a suspension of it injected into the skin of another normal rabbit. Dilutions of 1:10 to 1:1000 induced characteristic primary and secondary vaccinal lesions, although the same suspension in dilutions of 1:10 to 1:100 only caused the transitory "immediate reaction" after inoculation into a vaccine-immune animal. The young rabbit which had originally been castrated was injected endermically when 62 days old with active, controlled virus in dilutions of 1:100 to 1:1000. It proved completely resistant to infection.

b) Rabbit B was inoculated intracutaneously with virus 28 days before she gave birth to a litter of young. Characteristic vaccinal lesions resulted which were healed at the time of parturition. One of the young, a female, was inoculated cutaneously with virus at the age of 32 days. Lesions of vaccinal infection developed in dilutions up to 1:100,000. Two others of the litter, also females, were likewise found to be susceptible to vaccine-virus infection.

From this experiment it can be inferred that vaccine virus passes through the placenta of the rabbit and consequently can be found in the fetus. As in the case of man, the placental passage is not a constant phenomenon. The results support, therefore, the earlier observations of Ohtawara (22).

In one of the two tests just described, the virus was obtained from the testicle of one of the resistant young of a recently vaccinated mother. This young rabbit was itself resistant to vaccinal re-infection when tested more than two months after birth.

In the second test, dealing with a litter of three females from which no suitable tissues were taken for cataphoresis, all of the offspring were susceptible to vaccinal infection. In this case the mother was inoculated 28 days before the birth of the young.

DISCUSSION

The results of the experiments presented herewith would seem to suggest that recovery from vaccine-virus infection is followed by the

persistence of virus in the body for an indefinite period. During this period, the animal appears to be resistant to re-infection; on the other hand, when the virus eventually disappears from the tissues, immunity is lost. We have found that by applying cataphoresis to the tissues of animals long previously infected with vaccine virus and still resistant, we could recover the virus, whereas none could be obtained from rabbits similarly inoculated but no longer immune.

The results of recent experiments on virus-immune serum reactions are in line with the conception here discussed. The older view is that virus is destroyed by certain "virucidal" agents, or is completely eliminated from the body on recovery from infection. Recently, Todd, Andrewes, and ourselves (1, 23) have demonstrated that in some cases at least immune serum does not act either *in vivo* or *in vitro* in a manner to destroy virus. Now it has been shown as a corollary that a virus can be obtained from animals' tissues long after recovery from infection. The fact that up to the present time it has been impossible to secure active immunity against filterable-virus diseases unless living virus is used (24) can be viewed as corroboratory evidence. The unusual case of Lucas and Osgood (5) of a second attack of poliomyelitis in a child after two years can be explained as due to an absence of virus at that time in the patient, with consequent loss of immunity. The consensus of opinion is that man should be re-vaccinated within seven years because the immunity conferred by vaccination may be lost within this period. In the experiments here presented, certain rabbits failed to yield vaccine virus from their tissues after 133, 143, 170, 171, 175, and 178 days; and each of these animals was shown to be susceptible to re-infection.

CONCLUSIONS

1. By means of cataphoresis vaccine virus can be obtained from suspensions of tissue which are inactive by the usual tests of animal inoculation.

2. Active virus can be obtained by cataphoresis from the tissues of rabbits long recovered from the effects of cutaneous vaccination.

3. Evidence is brought forward which suggests that immunity in a virus disease, such as vaccinia, may be linked with the persistence in the body of living virus.

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THE EFFECT OF CATAPHORESIS ON POLIOMYELITIS VIRUS

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Recent investigations in the field of filterable viruses have been marked by a tendency to apply to the disease-producing agents of this class certain biological principles which govern the reactions of bacteria. Of particular interest have been the observations on the behavior of viruses in an electrical field (1, 2, 3). By means of cataphoresis of virus suspensions, it has been found that the effective agent could be revealed in tissues which, by the usual tests of animal inoculation, failed to show activity. This results from the migration of the virus, or possibly, of the material in a suspension carrying the virus, to one or the other pole, depending on the charge. Thus vaccine virus under ordinary conditions of hydrogen ion concentration migrates to the anode (2, 3). By this method, furthermore, vaccine virus has been isolated from rabbits wholly recovered from infection and several weeks or months after vaccination (3, 4). Finally, cataphoresis has been of use in separating vaccine virus from neutral virus-immune serum mixtures (5). Since these observations have a bearing on immunity in virus diseases, it was determined to ascertain the behavior of the poliomyelitic virus in an electrical field.

Method

The apparatus used was a modification of that described by Field and Teague (6) and by Todd (1). The details will be given by Olitsky and Long (4) in another communication. It is sufficient to say here that three large U tubes were employed, the combined capacity of which was about 96 cc. of fluid. The ends of the connecting tubes which dipped into the fluid under observation contained 1 per cent agar and 1 per cent NaCl. This furnished a convenient method for collecting the particles containing the virus, or the virus itself, which migrated with the current. From 114 to 117.5 volts and 0.8 to 4.2 milliamperes were passed for 3 hours through the fluid under examination between non-polarizable electrodes.

At the end of the period the agar plugs were removed under sterile conditions, ground in 1 cc. of Ringer's solution, and inoculated intracerebrally in monkeys.[1] The mode of preparation of the fluid will be given with each of the following experiments.

The first step was to determine whether the virus of poliomyelitis possessed the property of migrating in an electric field.

Experiment 1.—A piece of spinal cord of a monkey dead of typical experimental poliomyelitis was used. The nervous tissue was emulsified in 100 cc. of distilled water under aseptic conditions. The cataphoresis test.was done as follows: milliamperes current, 0.8; potential drop, 117 volts; time, 3 hours; pH of the suspension, 6.9. After 30 minutes the fluid at the cathodes was clear and that at the anodes cloudy. After 2 hours the cathodic material was quite clear for a distance of 2 cm. from the surface and the remainder was partly clear. The agar from the positive poles contained a diffuse whitish cloud and that from the negative pole was translucent. *Macacus rhesus* 1 was inoculated intracerebrally with 1 cc. of Ringer's solution containing the ground agar from the positive poles. On the ninth day after inoculation the animal was excited and tremulous and both legs were paralyzed. On the tenth day the prostrate monkey was etherized. The gross and microscopical lesions of the spinal cord and brain were typical of poliomyelitis. *Macacus rhesus* 2 received a similar intracerebral inoculation of Ringer's solution containing the agar from the negative poles of the same experiment. On the fifteenth day after inoculation all four limbs were paralyzed and the animal was prostrate. The monkey was etherized and the gross and microscopic lesions of the nervous system were typical of poliomyelitis.

From the fact that the animal inoculated with anodic material developed poliomyelitis first, we were led to suppose that the virus probably carried a negative charge. Since the monkey inoculated with the cathodic material also contracted the disease, although after a somewhat longer incubation period, it was thought that probably the virus suspension was too strong, and part of the virus adhered to the cathodic agar. To obviate this difficulty we next employed a Berkefeld "V" filtrate of a 5 per cent suspension of the active nervous tissue.

Experiment 2.—The spinal cord from a monkey with poliomyelitis was made into a 5 per cent suspension in distilled water. The emulsion was passed through a Berkefeld "V" candle and placed in the cataphoresis apparatus. The conditions

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[1] All experimental procedures on animals were made with the aid of deep ether anesthesia.

were: milliamperes, 0.8; potential drop, 116 volts; time, 3 hours. The pH of the filtrate was 6.5. Contrary to the previous experiment, in which suspension was used, the clearing in this case appeared at the positive pole and the cathodic material remained cloudy. *Macacus rhesus* 3 was inoculated intracerebrally with 1 cc. of Ringer's solution containing the ground agar from the positive pole. The fifth day after inoculation the animal was excited, weak, and ataxic. The next day a marked tremor and partial paralysis of all limbs were present. On the seventh day the animal was prostrate, and on the eleventh it was etherized. The lesions of the nervous system were typical of poliomyelitis. *Macacus rhesus* 4 (control) was inoculated the same day with the material from the negative pole. This monkey remained well throughout the period of observation.

It will be noted that the virus collected at the positive pole. It is of interest that the clouding due to migration of proteins in the fluid appeared at the pole opposite to the one at which the virus was found.

The next experiment was designed to recover by cataphoresis the virus from a non-infectious, neutral mixture of poliomyelitis virus and convalescent serum.

Experiment 3.—Poliomyelitis material of the pooled mixed virus strain which had been glycerolated for some time was made into a 5 per cent emulsion in salt solution and passed through a Berkefeld "V" filter. 50 cc. of the filtrate was mixed with an equal volume of human convalescent serum. 4 cc. of this mixture was inoculated intraspinally and intracerebrally in *Macacus rhesus* 5 (control). No symptoms developed. The material was therefore considered to have been neutralized. The remainder of the mixture was subjected to cataphoresis under the following conditions: milliamperes, 4.2; potential drop, 114 volts; time, 3 hours; pH = 6. *Macacus rhesus* 6 was inoculated intracerebrally with 1 cc. of Ringer's solution containing the agar from the positive pole. On the eleventh day after inoculation the animal was excited, slow in movement, and tremulous. Ptosis and a right facial paralysis were present. On the twelfth day the monkey was ataxic and partially paralyzed in all four limbs. The thirteenth day the prostrate animal was etherized. The gross and microscopic lesions in the spinal cord and brain confirmed the diagnosis of poliomyelitis. *Macacus rhesus* 7 (control) was inoculated intracerebrally on the same day with the emulsified agar from the negative pole of the same experiment. No symptoms developed.

The experiment shows unmistakably that active poliomyelitis virus can be separated from a non-infective, neutral mixture of the virus and its specific immune serum. We can thus conclude that the virus is not killed by the antiserum but only held in some sort of an ineffective combination. The result also confirms the previous obser-

vation that under the conditions of the experiment poliomyelitis virus bears a negative charge.

Since it was found possible to regain active poliomyelitis virus from the neutral mixture, it was thought advisable to attempt to isolate virus from an animal which had recovered from the disease.

Experiment 4.—A monkey, *Macacus rhesus* 8, was selected which had developed typical poliomyelitis 6 days after an intracerebral inoculation of virus. Almost complete paralysis of both arms and partial paralysis of the legs had existed for 4 days, followed by rapid recovery, so that by the eleventh day there was little disability. During the next 12 days the monkey ran and jumped about with almost normal vigor. At this time, or 23 days after infection, the animal was etherized and the spinal cord made into a 5 per cent suspension in salt solution. Cataphoresis was carried out under the following conditions: milliamperes, 0.8; potential drop, 117.5 volts; time, 3 hours; pH = 6.8. *Macacus rhesus* 9 was inoculated intracerebrally with the agar suspension from the positive pole. On the eighteenth day tremor, ataxia, and partial paralysis of both arms appeared. On the nineteenth the monkey was prostrate and was etherized. The gross and microscopic examination of the central nervous system confirmed the diagnosis of poliomyelitis. *Macacus rhesus* 10 (control) was inoculated intracerebrally with the material from the negative pole and 11 (control) with the suspension of central nervous system before cataphoresis had been performed. Neither animal developed poliomyelitis.

This experiment brings out the interesting fact that active virus persists in the nervous tissues for a period after recovery from poliomyelitis. Since ordinary inoculation of central nervous tissues before cataphoresis was without effect, the positive result indicates either that the antibody concentration in the tissues is sufficient to have neutralized the virus associated with it on injection in our experiment, or that in the recovered monkeys the virus is present in too minute quantity to be detected by the usual mode of inoculation. The tests described demonstrate that the virus of poliomyelitis may be separated in effective quantity from neutral mixtures of virus and immune serum or further that it can be concentrated at the positive pole from dilute virus filtrates.

CONCLUSIONS

1. Under ordinary conditions of hydrogen ion concentration the virus of poliomyelitis, as such, or associated with particles in fine sus-

pension, migrates in an electrical field to the anode.   It follows that the virus bears an electronegative charge.

2. By means of cataphoresis, the virus can be recovered from a non-infective mixture of virus and specific immune serum.

3. By the same means it is possible to reveal the presence of virus in the central nervous system of a monkey which has recovered from the active stage of experimental poliomyelitis.

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ADAPTATION OF MASTITIS STREPTOCOCCI TO MILK

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It is well known that fresh milk or milk heated at 58° or 60°C. for 20 minutes will inhibit the growth of a variety of organisms, while when milk is heated at a temperature of 80°C. or more the inhibitory principle is destroyed. That different streptococci behave differently when introduced into the same milk is brought out by the following observation: The growth of the nonhemolytic mastitis streptococcus is inhibited during the first 6 or 8 hours following inoculation and then growth begins and continues rapidly; but scarlet fever streptococci implanted in portions of the same milk gradually diminish in numbers until the milk finally becomes sterile. Both organisms grow readily in milk that has been boiled for 5 minutes.

It appeared to us that a more careful study of the end of the lag phase and the beginning of growth in the case of the mastitis streptococcus might throw some light on the relation between the inhibiting agent and the implanted organisms and perhaps help to explain the mode of action of the inhibitory substance. With these points in view a series of experiments was undertaken.

Experimental

Milk was obtained principally from two cows. Care was taken to prevent contamination by drawing the milk directly from the cleansed udder into sterile bottles. It was chilled at once, freed of fat by centrifugation, heated at 58°C. for 20 minutes, and refrigerated until used. As a rule it was 2 or more days old when inoculated. The culture usually employed was the nonhemolytic mastitis streptococcus which had been kept on artificial media. Subcultures for inoculation were made in broth and used after incubation at 38°C. for 16 hours. Further details are recorded in the protocols.

The effect of milk on the growth of the mastitis streptococcus is considered in Experiment 1.

Experiment 1. Proportionate quantities of milk heated at 58°C. for 20 minutes or boiled for 5 minutes were inoculated with the diluted broth culture of the mastitis streptococcus and incubated at 38°C. in a water bath. Portions were plated with 12 cc. of melted agar (veal infusion) immediately after inoculation and at intervals thereafter, and the colonies counted after 24 hours incubation at 38°C. Previous
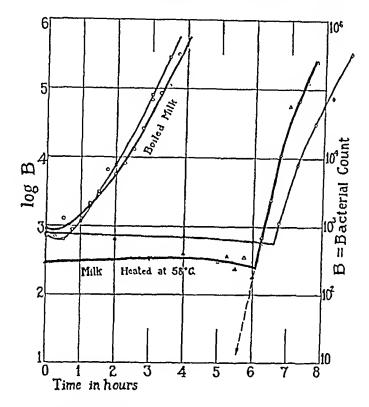
Fig. 1. Retardation of bacterial growth in milk previously heated at 58°C. for 20 minutes as compared with growth in boiled milk.

tests had shown that the lag period was relatively short in the sample which had first been boiled, so that platings were made at 15 minute intervals from the time of inoculation. The milk which had been heated at 58°C. was known to be inhibitory for this particular strain during the first 4 or 5 hours and it was only after 5 hours that frequent platings were made. The results are given in Table I and plotted in Fig. 1. They are typical for this organism under these conditions.

A similar experiment in which the intervals between platings were increased to 30 minutes showed substantially the same results, and the results have also been plotted in Fig. 1.

It will be noted from the data submitted that in the milk heated at 58°C. for 20 minutes the organisms tend to decline slightly in numbers during the first 6 hours. This phase is followed by a sharp "break" in which the organisms begin to multiply at a rapid rate and continue to do so throughout the period of observation. In the boiled milk the lag does not last longer than the first hour after which rapid growth

TABLE I

Growth of Mastitis Streptococcus in Milk

Milk boiled for 5 mins.			Milk heated at 58°C. for 20 mins.		
Time in hours after inoculation	B = bacterial count	Log B	Time in hours after inoculation	B = bacterial count	Log B
0	896	2.95	0	320	2.47
0.25	704	2.85	4.0	412	2.60
.50	1,280	3.11	5.0	307	2.48
.70	896	2.95	5.25	371	2.57
1.00	1,280	3.11	5.50	243	2.38
1.25	2,110	3.32	5.75	345	2.55
1.50	3,780	3.45	6.0	179	2.26
1.75	6,460	3.81	6.25	665	2.85
2.00	5,760	3.76	6.5	2,430	3.38
2.25	8,060	3.91	6.75	11,000	4.04
2.50	13,300	4.12	7.0	57,600	4.76
2.75	26,900	4.42	7.25	69,100	4.84
3.00	69,100	4.84	7.5	127,000	5.10
3.25	85,100	4.93	7.75	250,000	5.40
3.50	108,000	5.04	8.0		
3.75	313,000	5.50			
4.00	350,000	5.55			

begins. Repeated experiments have regularly shown that the onset of growth is sudden in milk heated at 58°C.

Three explanations to account for the sudden onset of growth in the milk previously heated at 58°C. for 20 minutes presented themselves: (1) That the inhibitory substance is utilized at some time during the lag period with the result that the uninhibited organisms grow rapidly; (2) that a resistant form is present from the start but only slowly multiplies to an appreciable number; and (3) that at some time during the lag period a resistant form develops which is capable of rapid

growth. The next three experiments were designed to test the importance of these factors.

TABLE II

Demonstration that the Inhibitory Substance Is Not Utilized during the Lag Phase
(B = bacterial count, *i.e.*, number of colonies per cc.)

A

Time in hours after inoculation	Boiled milk		Milk heated at 58°C.	
	B	log B	B	log B
0	384	2.58	358	2.55
2	4,740	3.68	409	2.61
4	86,000	4.93	499	2.70
5			1,340	3.13
5.5			1,730	3.24
5.75			2,500*	3.40*
6.0			3,700†	3.57†
6.25			9,340¶	3.97¶
6.5			26,600	4.43

B

Behavior of the Mastitis Streptococcus in Portions of A Removed at Various Times

Time in hours	5¾ hr. portion,* boiled		6 hr. portion,† 58°C.		6¼ hr. portion, ¶ 58°C.		Control portion reheated at 58°C. after 6 hrs. incubation	
	B	log B	B	log B	B	log B	B	log B
0	576	2.76	768	2.89	640	2.81	768	2.89
1	2,300	3.36	435	2.64	512	2.71	410	2.61
2	9,120	3.96	420	2.62	576	2.76	435	2.64
3	46,100	4.66	450	2.65	380	2.58	340	2.53
4	∞	∞	250	2.40	1,540	3.19	320	2.50
5	∞	∞	2,000	3.30	32,000	4.50	310	2.49
6	∞	∞	5,760	3.76	∞	∞	510	2.71

*†¶ The symbols indicate portions of A removed, heated and tested in B.

Experiment 2. Milk handled in the same manner as in Experiment 1 was divided into two lots. One lot, for control purposes, was boiled before inoculation and plated at 2 hour intervals during incubation at 38°C. The other lot was heated at 58°C. for 20 minutes, and likewise inoculated when cool. Portions of it were plated at 2 hour intervals for a time. The intervals were later decreased to

15 minutes. 10 cc. samples were removed after 5¾, 6, and 6¼ hours incubation. The first tube of inoculated milk, removed after 5¾ hours incubation, was boiled 5 minutes; and the other two, removed at 6 and 6¼ hours, respectively, were heated at 58°C. for 20 minutes to kill the implanted streptococci. A fourth tube of milk handled in the same manner but uninoculated was incubated for 6 hours and reheated at 58°C. It served as an additional control. All four samples were kept in the refrigerator overnight and in the morning were reinoculated with the culture and incubated at 38°C. Portions were plated each hour. The data are given in Table II and Fig. 2.
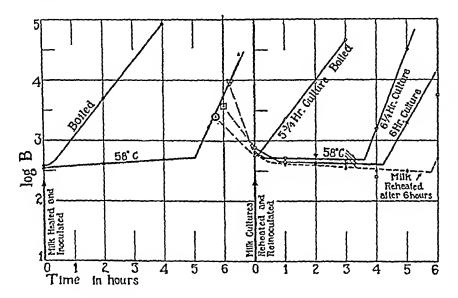
Fig. 2. Demonstration that the inhibitory agent is not utilized during the lag period.

It will be noted that the samples removed from the inoculated milk after 6 and 6¼ hours and then heated sufficiently to destroy the inoculated streptococci were still capable of producing considerable lag. It is evident that, though growth had taken place, the principle had been only slightly utilized. The contents of one of the control tubes inoculated and incubated for 5¾ hours and then boiled afforded a good culture medium from the first, whereas the action of the principle in the uninoculated milk had not been impaired either by incubation or repasteurization.

Since this experiment showed that the inhibitory substance had not been completely utilized, attention was turned to the possibility of an adaptation to the inhibitory substance on the part of the organism. The next two experiments furnished evidence compatible with the view that the organism adapts itself to the inhibitory principle.

TABLE III

A. *Effect of Fresh Milk on the Mastitis Streptococcus during the Lag Phase and the Beginning of the Growth Phase*

Time in hours	Boiled milk		58°C. milk	
	B	log B	B	log B
0	512	2.71	640	2.80
2	3,840	3.58	434	2.64
4	39,000	4.59	512	2.71
4.5	87,700	4.94	578*	2.76
5.5	495,000	5.70	1,540†	3.19
6.5	∞	∞	13,100¶	4.12
7.5	∞	∞	67,000§	4.83

B. *Subcultures Made by Centrifuging A and Exposing the Streptococci to the Action of Fresh Milk (Heated at 58°C.)*

Time in hours	4½ hr. culture*		5½ hr. culture†		6½ hr. culture¶		7½ hr. culture§	
	B	log B	B	log B	B	log B	B	log B
0	1,020	3.01	2,050	3.31	14,100	4.15	65,300	4.81
0.5	1,020	3.01	3,580	3.55	20,500	4.31	76,800	4.88
1	1,020	3.01	5,450	3.74	28,800	4.46	107,000	5.03
2	7,040	3.85	26,800	4.43	78,000	4.89	214,000	5.33
3	14,100	4.15	47,400	4.68	212,000	5.33	506,000	5.70
4	58,900	4.77	494,000	5.69	730,000	5.86	∞	∞

* † ¶ § These symbols indicate subcultures in B derived from the respective portions of A.

Experiment 3. 25 cc. of fresh milk which had been heated at 58°C. for 20 minutes, and 5 cc. of the same lot which had been boiled for 5 minutes were inoculated with proportionate quantities of, mastitis streptococci. Both tubes were incubated at 38°C. and portions plated at regular intervals. 5 cc. portions of the milk that had been heated at 58°C. were removed at 4½, 5½, 6½, and 7½ hours and centrifuged. The streptococci obtained from the sediment were mixed with

5 cc. of fresh milk previously heated at 58°C. and again incubated.   Care was taken not to chill the tubes during the process and the fresh milk was warmed to 38°C. before it was added.   Portions of each series were plated at intervals.   The results are given in Table III and Fig. 3.

It will be seen from the data that the period of inhibition extended to about the 5th hour in the milk that had been first heated at 58°C.,
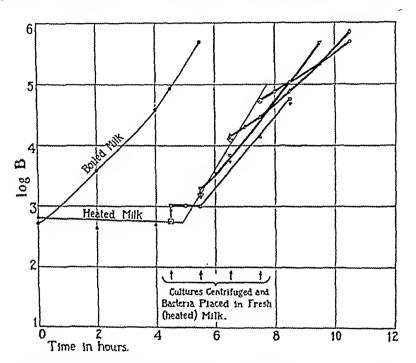
Fig. 3. Effect of fresh (pasteurized) milk on the rate of growth of mastitis streptococci removed from the original milk culture.

then the usual sharp break occurred and rapid growth ensued.   The bacteria removed about one half hour before the lag phase ended failed to multiply during the first hour of exposure in fresh milk but grew rapidly thereafter.   Thus in spite of the exposure to fresh milk the period of lag was about the same.   On the other hand the organisms removed during the active growth phase ($5\frac{1}{2}$, $6\frac{1}{2}$, and $7\frac{1}{2}$ hours after inoculation) all grew without lag in the fresh milk.

Experiment 4 shows that the streptococcus adapts itself to milk.

Experiment 4. Milk previously heated at 58°C. for 20 minutes was distributed in sterile tubes as follows: 1 tube containing 5 cc., and 4 tubes each containing 4 cc. To the tube containing 5 cc., 0.1 cc. of a 16 hour broth culture of the mastititis streptococcus was added and a sample plated at once to determine the number of streptococci. The tube was then placed in the water bath at 38°C. At the end of 1½ hours, 1 cc. was removed and added to one of the tubes containing 4 cc. of milk. A portion of the contents of the original tube was plated as was the

TABLE IV

Total time of incuba-tion in hours	Tube No.	Contents	Time of incuba-tion for each tube in hours	Colonies in plates $= \dfrac{BD}{625}$	Log $\dfrac{BD}{625}$	B = cal-culated bacteria per cc.
A. *Adaptation of the Mastitis Streptococcus to Frequently Added Milk*
0	1	0.1 cc. culture + 5 cc. milk	0	1,600	3.20	1,000,000
1½			1½	1,280	3.11	800,000
1½	2	1 cc. from Tube 1 + 4 cc. milk	0	1,088	3.04	136,000
3.0			1½	832	2.92	104,000
3.0	3	1 cc. from Tube 2 + 4 cc. milk	0	896	2.95	22,400
4½			1½	704	2.85	17,600
4½	4	1 cc. from Tube 3 + 4 cc. milk	0	768	2.87	3,840
6.0			1½	665	2.82	3,330
6.0	5	1 cc. from Tube 4 + 4 cc. milk	0	640	2.81	640
7½			1½	3,456	3.54	3,456
B. *Control Experiment with Boiled Milk*
0	1	0.1 cc. culture + 5 cc. boiled milk	0	1,280	3.11	800,000
1½			1½	10,300	4.01	6,440,000
1½	2	1 cc. from Tube 1 + 4 cc. boiled milk	0	11,300	4.05	1,410,000
3.0			1½	52,400	4.72	6,550,000
3.0	3	1 cc. from Tube 2 + 4 cc. boiled milk	0	57,000	4.76	1,430,000
4½			1½	∞	∞	∞

mixture in the second tube. After incubation for another 1½ hours, 1 cc. of the second tube was added to 4 cc. of milk, and plates were prepared as before. This procedure was repeated at the end of 4½ hours and 6 hours, so that the inoculated streptococci were regularly diluted and exposed to the action of fresh milk at intervals of 1½ hours. A control series was carried on in boiled milk. The plate cultures were always prepared with the same amount of fluid.

With respect to the first tube the five tubes had dilutions of 0, 1:5, 1:25, 1:125, and 1:625 respectively. In order that the bacterial counts might be comparable the milk was diluted with sterile NaCl solution before plating, as

follows: 1:625, 1:125, 1:25, 1:5, and 0, respectively, thus making all platings on a dilution of 1:625 of the mixture in the first tube.

If B is the bacterial count per cc. and D is the dilution (1, 5, 25, etc.), then BD/625 represents the count in each plate. These values are given in Table IV.

In spite of the fact that the mastitis streptococcus was exposed to the action of fresh milk at intervals of $1\frac{1}{2}$ hours it was able to establish
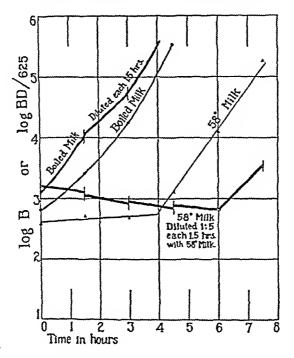
Fig. 4. Effect of milk added at frequent intervals on the growth of the mastitis streptococcus. The bacterial counts are corrected for the amount of dilution.

itself and begin to grow. The procedure, however, had two recognizable effects: first, it increased the lag period from about 4 hours in the milk control tube to about 6 hours in the experimental series, and further, there was a gradual but consistent decline in the number of streptococci until growth began. When the same experiment was done with boiled milk the streptococci increased throughout and the addition of fresh portions of boiled milk failed to retard growth.

Aside from the fact that the mastitis streptococcus becomes adapted to the inhibitory effect of milk the results in Experiment 2 indicated that a small portion of the principle had been utilized during the early phase of active growth. Experiments earlier reported[1] have shown that when milk is heavily seeded with bacteria and incubated sufficiently long to insure multiplication the inhibitory effect disappears. The question arises as to whether the utilization can be considered an adsorption by the bacterial cells. Experiment 5 is designed to throw light on this phase of the problem.

TABLE V

Bactericidal Activity of Milk Previously Treated with Living or Dead Scarlet Fever Streptococci

Time in hours	Milk heated at 58°C.		Milk incubated and refrigerated, then heated at 58°C.		Boiled milk (combined from all tubes)
	Incubated with dead streptococci, then centrifuged	Control milk	Incubated with live streptococci, centrifuged and heated at 58°C.	Control milk	
0	428	428	384	384	576
2	345	166	320	217	13,820
4	192	153	230	77	∞
6	12	4	12	2	∞
8	5	1	3	6	∞
24	0	0	0	0	
48	0	0	0	0	

Experiment 5. Mixed milk from five cows was chilled and freed from fat and 15 cc. amounts placed in four sterile tubes. Two of the tubes were heated at 58°C. for 20 minutes. One tube served as control, and to the other were added scarlet fever streptococci from 500 cc. of 48 hour broth culture which had been heated at 60°C. for 20 minutes. The two other tubes were not heated until after incubation. To one of these the scarlet fever streptococci from 500 cc. of 48 hour broth culture were added. All four tubes were incubated 2 hours and refrigerated 18 hours. The second pair of tubes, which had not been heated, were then heated at 58'C. for 20 minutes. All four tubes were centrifuged and the supernatant fluid was distributed into small tubes and reinoculated with scarlet fever streptococci. The results are given in Table V.

[1] Jones, F. S., and Little, R. B., *J. Exp. Med.*, 1927, 45, 319.

The experiment was also performed with large quantities of mastitis streptococci. In no case was the inhibitory substance appreciably removed by dead streptococci or by viable organisms, provided always that the tubes were not incubated sufficiently long to permit growth.

DISCUSSION

The experiments have shown that when mastitis streptococci are introduced in small numbers into milk previously heated at 58°C. for 20 minutes there is a lag in growth of 5 or 6 hours succeeded by an abrupt increase which continues at a rapid rate (Fig. 1). The experiments further indicate that the inhibitory principle is not destroyed during this period since sufficient remains to inhibit the growth of a similar culture for about 4 hours (Fig. 2). Furthermore had the substance been completely destroyed during the lag period the break in the curve would not have been as sharp.

The explanation that a resistant form is present from the start and requires a period of time to multiply to an appreciable number, can be immediately discarded from the shape of the curves in Fig. 1. Here we have plotted the logarithms of the bacterial count against time of incubation. Milk previously heated at 58°C. is shown to produce a long lag, followed by a sharp break and rapid growth. The experiment was performed enough times and at intervals sufficiently close for us to be certain of the abruptness of the change. Even if only one bacterium of a strain resistant to the inhibitory agent had been present at the time of inoculation, this one bacterium, if it multiplied at the rate shown to be typical of the surviving forms, could have produced a lag of not more than 3 hours. This calculation takes into account the normal lag of 1 hour which is found when the streptococcus is grown in boiled milk. Thus, if we take the curve after the lag period and extrapolate it to zero time, as indicated by the dash line in Fig. 1, we find that if the surviving bacteria were grown from a single resistant organism this organism was not produced until the 5th hour.

The findings justify us in concluding that mastitis streptococci become adapted to the inhibitory agent. Further proof of this conclusion is given by Experiment 3 in which bacteria surviving the lag period are found to grow rapidly, without lag, when placed in fresh

milk. From Fig. 3 it will be seen that mastitis streptococci removed from milk cultures after various periods of incubation and placed in fresh milk behave much as if they had been left in the original culture. The $4\frac{1}{2}$ hour sample had not reached the period of rapid growth and showed a short lag in fresh milk. The streptococci removed after rapid growth showed no lag.

The results of the experiment cited raise the question as to what would occur if the bacteria were frequently placed in fresh milk during the period of incubation. This was tested in Experiment 4, the conditions of which resemble in some respects those found in the udder. In this experiment a milk culture of mastitis streptococci was diluted 1:5 with fresh (pasteurized) milk each $1\frac{1}{2}$ hours. The tabulated bacterial count (B) is corrected for the dilution (D) so that the numerical value of BD/625 represents the bacteria per cc. for all cultures, on the basis of the dilution in the last culture.   Fig. 4 shows that in spite of changing the milk each $1\frac{1}{2}$ hours, the streptococcus develops a resistant type in 6 hours, whereas a lag of 4 hours occurred in the control tube the milk of which was not changed. As a result of the repeated additions of milk there was approximately a 25 per cent mortality among the streptococci. When the experiment was performed with boiled milk as a medium, rapid multiplication took place in spite of the frequent dilutions.

It has been shown[2] that scarlet fever streptococci die in milk. Their numbers gradually decline during the first 24 hours and finally the milk becomes sterile. The fact that the effect is not one of lysis is readily determined by microscopic examination, since intact streptococci may be demonstrated in the milk. It would seem that the action of milk against both the bovine and the scarlet fever streptococcus involves a factor which prevents multiplication. In the case of the mastitis streptococcus cell division is prevented for a time but a resistant type that is capable of multiplication develops. The scarlet fever streptococcus is unable to produce resistant forms and hence perishes.
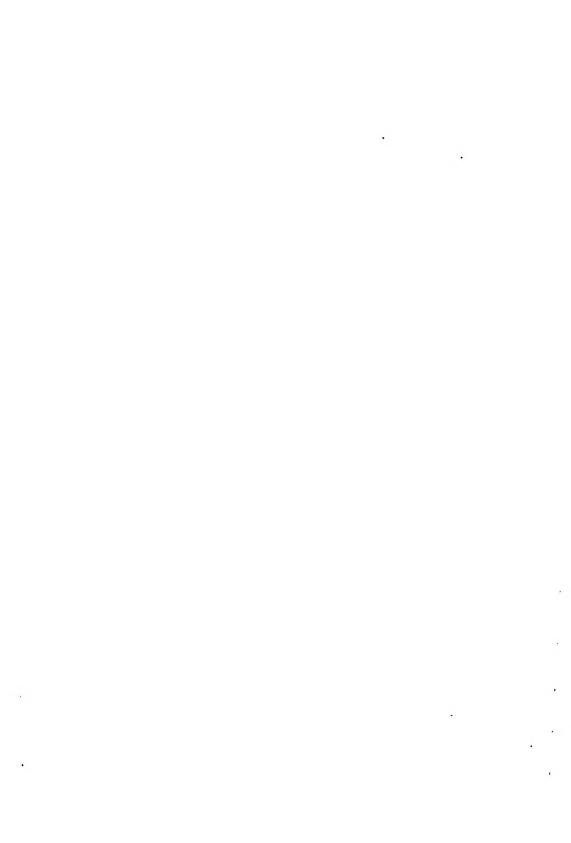
Experiment 5, in which we attempted to adsorb the substance with large numbers of dead or living streptococci, indicates that

[2] Jones, F. S., *J. Exp. Med.*, 1928, **47**, 965.

there is no specific union between bacterial cell and the substance, since the inhibitory character of the milk was not diminished by such treatment.   It seems probable that the substance is one preventing multiplication of bacterial cells and is not notably toxic in itself.

SUMMARY

The data here presented indicate that the inhibitory principle affecting the growth of streptococci in milk is not greatly utilized during the lag phase and that the abrupt termination of lag is not due to the utilization of the principle.   They further indicate that the sudden beginning of growth cannot be ascribed to a resistant type of streptococcus present in the culture from the first, but to an adaptation occurring during the lag phase.   The addition of large numbers of dead or living streptococci to milk, for limited periods, fails to diminish appreciably the inhibitory principle.

THE LESIONS IN THE SKELETAL MUSCLES IN EXPERIMENTAL SCORBUTUS

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PLATE 13

This report describes a lesion observed in the skeletal muscles of scorbutic guinea pigs and seeks to clarify its relationship to the scorbutic process as well as to demonstrate a factor modifying its distribution.

The voluminous literature bearing on scorbutus contains relatively little reference to the skeletal muscles. With two exceptions those authors who have described muscle lesions have merely mentioned that thinning and waxy degeneration of the fibers are occasionally to be found. Reports of degeneration of the muscles occur in the early studies of the disease in man by Hayem (1), Nambu and Sato (2), and recently in a study of experimental scurvy by Meyer (3).

The first report to emphasize the importance of the lesions in muscles was the monograph of Aschoff and Koch (4), containing observations of the disease in Balkan soldiers during the recent war. These authors studied the thigh muscles but were primarily interested in the hemorrhages, and chose hemorrhagic areas for study. They found changes similar to those previously reported by other workers. They believed the hemorrhages were largely responsible for the degenerative change and the thinning and atrophy the result of the cachexia in their cases.

Subsequently Höjer (5) described lesions in the muscles in experimental scorbutus which he regarded as an intrinsic part of the disease. His observations conform with our own on several points.

The material on which this report is based consists of seventy-one guinea pigs of which forty-eight were scorbutic. Many of the animals were studied in coöperation with Dr. Walter H. Eddy of Teachers College, Columbia University, who was conducting feeding experiments, and these were partially protected with canned vegetable or fruit. Most of the animals were submitted to us for diagnosis, without

record of their weight curve, behavior or diet. In some cases only selected tissues were received. The examination was chiefly of ribs and long bones, where characteristic changes occur in scurvy.

The diagnosis of scurvy is not difficult. The criteria employed were cessation of bone growth, irregularities, spreading and fragmentation of the cartilage columns and the development of the so-called "gerüstmark," a lax, delicate connective tissue which appears at the junction of bone and cartilage. Other elements in the histo-anatomical picture are hemorrhages and separation of bone and cartilage or pathological fracture of bone. The lesions are not uniformly disposed and many samples are at times necessary to estimate properly the extent of the disease or even its presence. The latter fact is especially true in animals which receive a diet only partially deficient in anti-scorbutic material.

In our routine examination of the ribs we commonly found a lesion of the intercostal muscles. Its nature varied with the characteristics of the bone lesion.

When the disease is very marked and active in the bones one finds that a variable number of muscle cells have become opaque, strongly eosinophilic and fragmented. The lines of recent fractures run directly across the fibers, paralleling the striations. Usually, however, the striations have disappeared. The segments of the muscle cells may be rectangular, square or discoid. In the latter case a series of these discoid fragments resembles blood rouleaux. Some muscle cells have been seen in which there were regular, deep clefts on only one side of the cell. The degenerate portions lose their affinity for acid dyes and appear swollen. Some show crevices and loculi within. These fibers are surrounded by large endothelial cells which engulf them and form foreign body giant cells.

Two other kinds of cells may be distinguished. Rows of spindle shaped fibroblasts with delicate fibroglia form between the muscle cells and large cells with clear acidophilic cytoplasm appear, singly and in clusters, near the point of fracture and even between the muscle fibers. These latter cells resemble young muscle cells and many are in process of mitotic division. A further study of these cells in under way to determine if possible their exact nature.

In chronic scorbutus, that is, where the lesion in the bone appears

inactive and the diseased costo-chondral junction is largely fortified by a connective tissue scar, and the "gerüstmark" has become passive, its cells no longer dividing actively, the muscles present a different picture. In such cases densely stained hyaline fragments, few in number, will be found lying in a lax, collagen-poor connective tissue. This stroma is identical with the "gerüstmark" in its cellular structure with the single exception that in the latter case dead islands of calcified cartilage or other remnants of past osteoid function are embedded within it, while in the former, fragments of muscle fibers remain.

It will be noted that in many features the lesion described resembles hyaline or Zenker's degeneration as seen in infectious and toxic diseases. Indeed the resemblance to that variety of degeneration as Forbus (6) has found it in guinea pigs is very close. Our animals were carefully searched for evidence of infectious disease but in none of those appearing in this series was there reason to suspect infection.

After some experience with this material it appeared that animals with scorbutic changes in the bones invariably presented lesions in the muscles. Indeed, in cases where the first preparations showed muscle involvement but no bone changes, further sampling regularly disclosed the presence of lesions in other bones. The intercostal muscles were invariably involved. A search for similar changes in the extensors of the thighs and forelegs and in the psoas muscle disclosed no comparable changes. Only an occasional darkly stained muscle cell was seen. It is of interest to note that in a general way within the intercostal muscles the lesions tended to favor certain locations. They were most frequent near the junction of cartilage and bone, or when present throughout the muscle they were more pronounced in these regions.

Several facts suggest that the muscle lesion is an intrinsic part of the scorbutic process. It occurs regularly in the scorbutic animals and never in the controls. It bears a definite and constant relationship to the scorbutic lesion in the bones, being active when bone changes are active, and inactive when the bone lesions are inactive. Moreover, we have not been able to find any other cause for the condition.

The selectivity of the lesion for the thoracic wall seemed to call for an explanation. It was suspected that possibly another factor other than the scurvy determined the location of the lesion and that this factor might be the activity of the thoracic muscles. The weakened

scorbutic guinea pig spares the muscles of the skeleton except those necessary to vital functions, as the respiratory muscles. If the conception just mentioned is correct the diaphragm and possibly the masseter muscles should show some degeneration as well as the intercostals. In most of our specimens these muscles had not been preserved but of eight cases in which material was available for examination the masseter muscles were found to be degenerate in three instances and the diaphragmatic muscles in five.

To further test this point of view several animals with advanced scorbutus were placed in a large barrel which could be rotated slowly with a motor so that the animals were required to exert themselves to stay on their feet. The animals were given 1 hour of this exercise every day and were then sacrificed. In each instance, florid, extensive lesions were found in the several muscles of the hind and fore legs and psoas muscles. In five non-scorbutic animals no muscle lesions were found. In all, seven scorbutic guinea pigs have been so tested. The records of three are given below.

DISCUSSION

The earliest clinical evidence of scurvy in a guinea pig is a change in attitude. At times this is represented by the elevation and sparing of one leg or by a tendency to squat quietly for long periods. Later in the disease the animals become nearly helpless and may lie on one side, apparently unable to move. In human cases an early sign of the disease (7) is loss of vigor with a tendency to tire quickly. Scorbutic infants exhibit marked tenderness in their thighs and commonly keep their extremities slightly flexed. These well known phenomena have been explained as the result of hemorrhages. The tenderness in the thighs of scorbutic infants has been said to be the result of subperiosteal hemorrhages. Observation of our guinea pigs suggests that muscle lesions may be largely responsible for both the tenderness and weakness. Moreover we have seen tenderness and the characteristic muscle degeneration develop in muscles which had been bruised.

The selective development of the muscle degeneration at points of exercise and trauma is in accord with other features of scorbutus. A causal relationship exists in the case of the hemorrhages. As was

emphasized by Aschoff and Koch, hemorrhages occur in places exposed to trauma. Bleeding about vessels and nerves was noted by these authors only when the vessel or nerve was in an exposed location, never when it was protected by bone.

A less marked relationship exists in the case of the bone lesions but here a causal connection between stress and lesion is suggested. In the thoracic wall, for example, lesions are most common in the more active and exposed ribs being relatively uncommon in the first two or three of these bones. I have noted that guinea pigs kept in separate cages, which remain quiet throughout the disease, show less marked lesions than animals which have been kept active by crowding or excitement. A special observation of the sort was made in the case of six animals sent to us while alive and which had been necessarily much shaken in transit. Some of the most striking muscle and bone lesions were present in this group.

Wolbach and Howe (8) believe that the underlying change in the bones is a deficiency in the intercellular substances. Aschoff and Koch and others have supposed that the hemorrhages were the result of altered cement substances in the vessel walls which predispose to rupture or leakage. We have noted rupture in the striated muscle. Whether a related underlying cause is responsible is not apparent from our material. We have seen cells which had ruptured but which appeared otherwise normal. If degeneration of the muscle fiber does not precede separation it follows it closely, for the usual fragmented cell is hyaline.

CONCLUSIONS

1. Muscle degeneration was constantly found in the intercostal muscles of scorbutic guinea pigs.

2. It has likewise been found in the masseter and diaphragmatic muscles.

3. Exercise will produce an identical lesion in other skeletal muscles in scorbutic animals.

4. The lesions appear to be an intrinsic part of the scorbutic process.

5. It is suggested that the tenderness over muscles in scorbutic animals and in man may be due to this myopathy.

Specimen Histories of Exercised Animals

Animal A.—Had been on basal diet plus 2 gm. canned tomato for 90 days. The weight at the beginning of the experiment was 315 gm. The weight on the 90th day was 422 gm. On the 91st and 92nd days the animal was exercised ½ hour, on the following 4 days 1 hour each day. The following day the animal was sacrificed. There was much pale fat throughout. The joints were easily dislocated, the bone crackled like egg shell, the teeth were loose. The costochondral junctions showed broad zones of "gerüstmark." A few, small, superficial hemorrhages were present in the muscles of the extremities. In the microscopic preparations all of the muscles of the extremities as well as the psoas and intercostal muscles showed extensive and severe degeneration.

Animals B and C.—Were practically identical. They were receiving 2 gm. canned peas daily and had gained weight. Both had the attitude of scorbutic animals and had joint tenderness. Each was exercised on 3 successive days for 1 hour per day. In both there was widespread muscle degeneration and abundant evidence of scorbutus in the bony system.

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EXPLANATION OF PLATE 13

Fig. 1. Degeneration in an intercostal muscle with active proliferation of young muscle cells (?). Two of these cells are in process of mitotic division. Many fibroblasts and endothelial cells are likewise present.

Fig. 2. A late stage in muscle degeneration in scorbutus. A few muscle fiber fragments lie in a collagen poor connective tissue stroma.
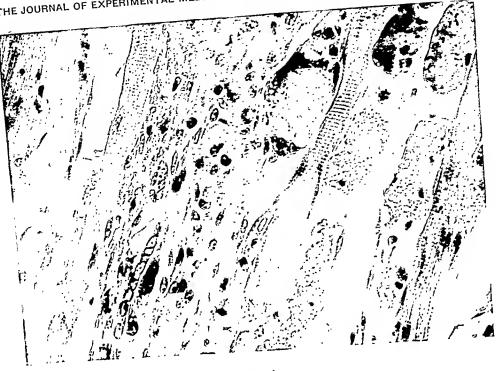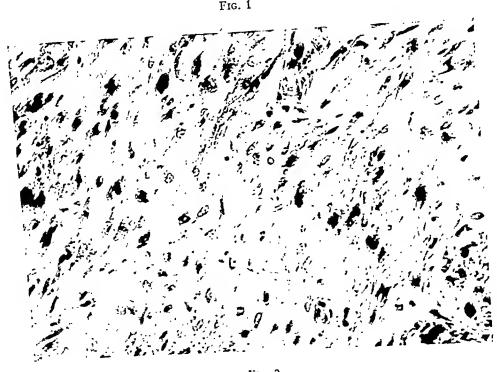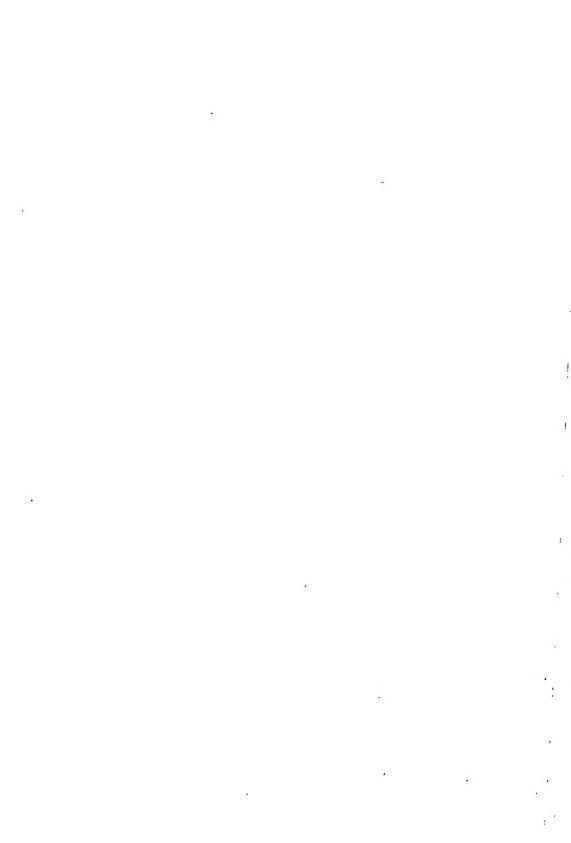
FIG. 1

FIG. 2

(Dalldorf: Lesions in experimental scorbutus)

THE GERMICIDAL ACTION OF HYDROXY SOAPS

By ARNOLD H. EGGERTH, Ph.D.*

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This paper is the fourth of a series of papers dealing with the germicidal action of soaps and soap derivatives. In the present investigation, the α-hydroxy soaps, and the soap of ricinoleic acid, were prepared and tested.

Preparation of Hydroxy Fatty Acids

The α-hydroxy fatty acids are prepared by heating the α-brom fatty acids with an excess of dilute aqueous sodium hydroxide. This is best done by heating in the Arnold steam sterilizer for several hours. The bromine is split off by the alkali, forming sodium bromide and the hydroxy soap. A small amount of the alpha unsaturated soap is likewise formed. The hydroxy acid is liberated with dilute sulphuric acid, filtered, washed with water, and dried. One has a choice of several satisfactory methods of purification: the most convenient is to dissolve the product in a small quantity of ether and then add a large excess of petroleum ether, which precipitates the hydroxy acids. This process is repeated until a constant melting point is obtained. Further details for the preparation and purification of the various hydroxy acids are given by the following authors: α-hydroxybehenic acid, by Fileti (1907); α-hydroxystearic, -palmitic, and -myristic acids, by Le Sueur (1905); α-hydroxylauric acid, by Guerin (1903); α-hydroxycapric acid, by Bagard (1907); and α-hydroxycaprilic acid, by Ley (1877). The hydroxy acid with 20 carbon atoms was not prepared. (In the preceding investigation (Eggerth, 1929), the α-bromoarachidic acid was prepared and tested, on the assumption that it was a normal acid having 20 carbon atoms. The writer at that time was not acquainted with the work of Ehrenstein and Stuewer (1922) who have shown that arachidic acid is actually iso-behenic, with 22 carbon atoms. It is interesting to note that the α-bromoarachidic soap actually gave germicidal titers that were in every case practically the same as those of the α-bromobehenate.)

The α-hydroxy fatty acids have higher melting points and are stronger acids than the parent unsubstituted acids. Their soaps are somewhat less hydrolysed by water. Nevertheless, those soaps having 14 or more carbon atoms are strongly alkaline to phenolphthalein. The sodium soaps are very little soluble in water; the potassium soaps are much more so.

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Ricinoleic acid is a normal fatty acid having 18 carbon atoms, with a double bond between the 9th and 10th carbon atoms, and a hydroxyl group attached to the 12th carbon atom. It is a hydroxylated oleic acid. It seemed desirable to study it here, inasmuch as it is the only member of its series that is known and available. It was prepared from castor oil by saponification; the liberated crude acid was thoroughly extracted with petroleum ether, converted to the barium salt, recrystallized several times from alcohol, and liberated with sulphuric acid.

Parallel tests were made with soaps of saturated unsubstituted fatty acids and oleic acid. The former were purchased from the Eastman Kodak Co.; the latter was Kahlbaum's best grade of oleic acid.

Technic of Germicidal Tests

The potassium soaps were made by adding the theoretical amount of KOH solution to weighed quantities of the fatty acids. Serial dilutions were made in sterile distilled water just prior to making the germicidal tests.

Buffer solutions set at pH 6.5, 7.5, and 8.5 were prepared. These contained $N/10$ potassium phosphate and $N/20$ glycine; details of their composition are given in a previous paper (Eggerth, 1926). When the test organism was *Diplococcus pneumoniæ*, the buffer solution was made to contain $N/20$ phosphate. The sterilized buffer fluids were inoculated with the test organisms, and 0.5 cc. quantities were pipetted into series of small test tubes; to these, 0.5 cc. quantities of the soap dilutions were added and the contents of the tubes well mixed. Thus the final concentration of potassium phosphate was $N/20$, except in the case of *Diplococcus pneumoniæ*, where the final concentration was $N/40$. (*Diplococcus pneumoniæ* frequently failed to survive for 18 hours in the $N/20$ potassium phosphate controls, whereas it always survived in the $N/40$ phosphate.) The tests were incubated at 37°C. and one loopful subcultured on glucose blood agar plates at the end of 30 minutes, 2 hours, and 18 hours.

The following test organisms were used: *Diplococcus pneumoniæ; Streptococcus hæmolyticus; B. diphtheriæ; Staphylococcus aureus; Micrococcus ovalis; B. typhosus; Vibrio choleræ; B. lepisepticus; B. melitensis; B. pyocyaneus.* With the exception of the last, these organisms are the same strains as those used in previous investigations with soaps (Eggerth, 1926, 1927, 1929).

The inoculations in the germicidal tests were such that each cubic centimeter of final test fluid contained 0.02 cc. of a 24 hour broth culture, with the exception of *Staphylococcus aureus*, *B. typhosus*, and *B. pyocyaneus*, where the inoculum was 0.01 cc.

DISCUSSION

As with other soaps previously reported, the germicidal titers of the α-hydroxy soaps increase with increasing molecular weight to a

TABLE I

The Germicidal Titers of α- Hydroxy-Soaps

		α-hy-droxy-capry-late	α-hy-droxy-caprate	α-hydroxy-laurate	α-hydroxy-myristate	α-hydroxy-palmitate	α-hydroxy-stearate	α-hydroxy-behenate
No. of carbon atoms		8	10	12	14	16	18	22

Diplococcus pneumoniæ

Time	pH							
2 hrs.	6.5	0	N/160	N/2560	N/20,480	N/163,840	N/163,840	
	7.5	0	N/40	N/320	N/2560	N/10,240	N/5120	0
	8 5	0	N/40	N/320	N/2560	N/10,240	N/2560	
18 hrs.	6.5	N/20	N/640	N/5120	N/81,920	N/327,680	N/327,680	
	7.5	N/20	N/80	N/1280	N/5120	N/40,960	N/40,960	N/20,480
	8.5	N/20	N/80	N/1280	N/2560	N/40,960	N/20,480	

Streptococcus hæmolyticus

2 hrs.	6.5	0	N/20	N/320	0	0	0	
	7.5	0	N/20	N/160	N/320	N/160	N/80	0
	8.5	0	0	N/160	N/640	N/160	N/160	
18 hrs.	6.5	0	N/320	N/640	N/2560	N/5120	N/640	
	7.5	0	N/40	N/640	N/1280	N/1280	N/640	N/640
	8.5	0	N/40	N/640	N/1280	N/1280	N/640	

Staphylococcus aureus

2 hrs.	6.5	0	0	N/2560	N/10,240	N520	N/640	
	7.5	0	N/10	N/160	{N/80, N/20,480}	N/80	N/80	0
	8.5	0	0	N/40	N/320	N/160	N/80	
18 hrs.	6.5	0	N/40	N/5120	N/40,960	N/20,480	N/1280	
	7.5	0	N/20	N/640	{N/160, N/20,480}	{N/160, N/10,240}	N/1280	0
	8.5	0	0	N/160	N/640	N/1280	N/640	

B. diphtheriæ

2 hrs.	6.5	0	N/160	N/2560	N/20,480	N/20,480	N/320	
	7.5	0	N/20	N/160	N/80	N/80	N/80	0
	8 5	0	N/20	N/160	N/320	N/640	N/320	
18 hrs.	6 5	0	N/640	N/10,240	N/81,920	N/81,920	N/40,960	
	7 5	0	N/80	N/1280	N/20,480	N/40,960	N/20,480	0
	8 5	0	N/20	N/320	N/2560	N/5120	N/1280	

TABLE I—*Continued*

		α-hydroxy-caprylate	α-hydroxy-caprate	α-hydroxy-laurate	α-hydroxy-myristate	α-hydroxy-palmitate	α-hydroxy-stearate	α-hydroxy-behenate
No. of carbon atoms		8	10	12	14	16	18	22

Micrococcus ovalis

Time	pH							
2 hrs.	6.5	0	0	N/160	0	0	0	
	7.5	0	0	N/80	N/80	0	0	0
	8.5	0	0	N/80	N/160	N/320	0	
18 hrs.	6.5	0	N/40	N/320	N/1280	N/1280	0	
	7.5	0	N/20	N/160	N/160	N/320	0	0
	8.5	0	0	N/160	N/640	N/1280	0	

Vibro cholerae

Time	pH							
2 hrs.	6.5	0	N/160	N/2560	N/5120	N/320	0	
	7.5	0	N/40	N/320	N/1280	N/640	0	0
	8.5	0	N/40	N/160	N/1280	N/640	N/160	
18 hrs.	6.5	N/20	N/640	N/5120	N/5120	N/640	0	0
	7.5	0	N/40	N/640	N/2560	N/640	N/160	0
	8.5	0	N/40	N/640	N/2560	N/640	N/160	

B. lepisepticus

Time	pH							
2 hrs.	6.5	0	N/320	N/5120	N/20,480	N/5120	N/320	
	7.5	0	N/80	N/320	N/1280	N/2560	N/640	N/160
	8.5	0	N/80	N/160	N/1280	N/5120	N/640	
18 hrs.	6.5	N/40	N/640	N/10,240	N/81,920	N/10,240	N/2560	
	7.5	N/20	N/320	N/1280	N/2560	N/2560	N/1280	N/640
	8.5	N/10	N/160	N/640	N/2560	N/5120	N/2560	

B. melitensis

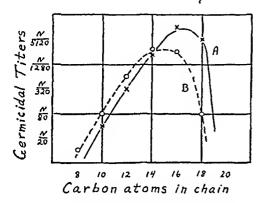| Time	pH							
2 hrs.	6.5	0	N/80	N/320	N/2560	0	0	
	7.5	0	N/40	N/160	N/1280	N/80	0	0
	8.5	0	N/40	N/160	N/1280	N/320	0	
18 hrs.	6.5	N/20	N/160	N/2560	N/5120	N/5120	0	
	7.5	N/10	N/160	N/640	N/1280	N/160	0	0
	8.5	N/10	N/80	N/320	N/1280	N/1280	0	

TABLE I—*Concluded*

		α-hydroxy-caprylate	α-hydroxy-caprate	α-hydroxy-laurate	α-hydroxy-myristate	α-hydroxy-palmitate	α-hydroxy-stearate	α-hydroxy-behenate
No. of carbon atoms		8	10	12	14	16	18	22
B. pyocyaneus								
Time	pH							
2 hrs.	6.5	0	0	0	0	0	0	
	7.5	0	0	N/80	N/160	N/80	0	0
	8.5	0	0	N/80	N/640	N/1280	0	
18 hrs.	6.5	0	0	0	0	0	0	
	7.5	0	0	N/80	N/160	N/80	0	0
	8.5	0	0	N/80	N/2560	N/1280	0	
B. typhosus								
2 hrs.	6.5	0	N/40	N/160	0	0	0	
	7.5	0	N/10	N/160	N/640	N/80	0	0
	8.5	0	N/40	N/160	N/1280	N/320	0	
18 hrs.	6.5	0	N/40	N/320	0	0	0	
	7.5	0	N/40	N/160	N/640	N/160	N/80	0
	8.5	0	N/40	N/320	N/2560	N/1280	N/160	

The lowest dilutions tested were N/10 for the hydroxycaprylate and hydroxy-caprate, N/40 for the hydroxylaurate, and N/80 for the others. Temperature, 37°C.

given maximum, and then diminish. (Table I and Figs. 1 and 2.) With all organisms, this maximum occurs either with the a-hydroxy-myristate or the -palmitate; there is no sharp differentiation between Gram positive and Gram negative bacteria as was the case with the α-brom soaps (Eggerth, 1929).

The germicidal action of the α-hydroxy soaps varies a great deal with the different test organisms (see Table I). The α-hydroxycapry-late does not kill any organism in a concentration of N/10 in 2 hours; in 18 hours, slight germicidal action is manifested towards *Diplococcus pneumoniæ*, *B. melitensis*, and *B. lepisepticus*. The α-hydroxy-caprate is more active, especially toward *Diplococcus pneumoniæ*, *B. melitensis*, *B. lepisepticus*, *Vibrio choleræ*, and *B. typhosus;* there is no action upon *B. pyocyaneus*, and very little upon *Staphyloccus aureus* and *Micrococcus ovalis*. The α-hydroxylaurate acts more

uniformly toward the ten test organisms than the other soaps of this series; the highest titers at pH 7.5 for 2 hours being N/320 and the lowest, N/80. Germicidal titers increase with the α-hydroxy-myristate, though very unequally. As we pass to the α-hydroxypalmitate, we find that the titers are increased with *Diplococcus pneumoniæ* and *B. lepisepticus*, but diminished with *Streptococcus hæmolyticus, Vibrio choleræ, B. melitensis; B. pyocyaneus*, and *B. typhosus.* The α-hydroxystearate is still highly germicidal for *Diplococcus pneumoniæ, B. diphtheriæ*, and *B. lepisepticus;* its titer for the other organisms is

FIG. 1. The germicidal titers of α-hydroxy soaps (*A*) and of unsubstituted saturated soaps (*B*) for *Diplococcus pneumoniæ*, at pH 7.5. The soaps are designated by the number of carbon atoms in their molecule. Time of test, 2 hours; temperature, 37°C.

FIG. 2. The germicidal titers of α-hydroxy soaps (*A*) and of unsubstituted saturated soaps (*B*) for *Vibrio choleræ*, at pH 7.5. The soaps are designated by the number of carbon atoms in their molecule. Time of test, 2 hours; temperature, 37°C.

very much diminished. Curiously enough, the only germ to be killed by the α-hydroxybehenate in 2 hours was *B. lepisepticus;* in 18 hours, this soap was toxic for three species: *Diplococcus pneumoniæ, Streptococcus hæmolyticus*, and *B. lepisepticus.*

The specific effect of the hydroxyl group upon the germicidal titer may be seen on comparing Table I with Table II, and in Figs. 1 and 2. In many cases, though by no means all, the germicidal titers of an α-hydroxy soap are very nearly the same as that of an unsubstituted soap having two less carbon atoms. This is particularly true of the titers for *Vibrio choleræ, B. melitensis, B. lepisepticus*, and *Strepto-*

TABLE II

The Germicidal Titers of Unsubstituted Soaps, at pH 7.5

	Caproate	Caprylate	Caprate	Laurate	Myristate	Palmitate	Stearate
No. of carbon atoms	6	8	10	12	14	16	18

Diplococcus pneumoniæ

	Caproate	Caprylate	Caprate	Laurate	Myristate	Palmitate	Stearate
2 hrs.	0	N/10	N/80	N/640	N/2560	N/2560	N/80
18 "	N/10	N/20	N/160	N/1280	N/10,240	N/10,240	N/160

Streptococcus hæmolyticus

	Caproate	Caprylate	Caprate	Laurate	Myristate	Palmitate	Stearate
2 hrs.	0	N/10	N/40	N/320	N/640	N/160	0
18 "	0	N/20	N/80	N/640	N/1280	N/640	0

Staphylococcus aureus

	Caproate	Caprylate	Caprate	Laurate	Myristate	Palmitate	Stearate
2 hrs.	0	0	N/40	N/320	N/160	0	0
18 "	0	0	N/40	N/320	N/640	0	0

B. diphtheriæ

	Caproate	Caprylate	Caprate	Laurate	Myristate	Palmitate	Stearate
2 hrs.	0	0	N/40	N/640	N/320	N/80	0
18 "	0	N/10	N/160	N/1280	N/5120	N/80	N/80

Micrococcus ovalis

	Caproate	Caprylate	Caprate	Laurate	Myristate	Palmitate	Stearate
2 hrs.	0	0	N/20	N/80	N/40	0	0
18 "	0	0	N/80	N/320	N/40	0	0

Vibrio choleræ

	Caproate	Caprylate	Caprate	Laurate	Myristate	Palmitate	Stearate
2 hrs.	0	N/20	N/160	N/640	N/160	0	0
18 "	N/20	N/20	N/320	N/640	N/320	0	0

B. lepisepticus

	Caproate	Caprylate	Caprate	Laurate	Myristate	Palmitate	Stearate
2 hrs.	0	N/10	N/160	N/1280	N/2560	N/160	0
18 "	0	N/40	N/640	N/10,240	N/10,240	N/320	0

B. melitensis

	Caproate	Caprylate	Caprate	Laurate	Myristate	Palmitate	Stearate
2 hrs.	0	N/10	N/160	N/1280	N/320	0	0
18 "	0	N/20	N/160	N/2560	N/1280	0	0

TABLE II—*Concluded*

	Caproate	Caprylate	Caprate	Laurate	Myristate	Palmitate	Stearate
No. of carbon atoms	6	8	10	12	14	16	18
B. pyocyaneus							
2 hrs.	0	0	0	$N/20$	$N/40$	0	0
18 "	0	0	0	$N/20$	$N/40$	0	0
B. typhosus							
2 hrs.	0	$N/10$	$N/40$	$N/160$	0	0	0
18 "	0	$N/10$	$N/80$	$N/160$	0	0	0

The lowest dilutions tested were $N/10$ for the caproate, caprylate, and caprate, $N/20$ for the laurate, $N/40$ for the myristate and $N/80$ for the palmitate and stearate. Temperature, 37°C.

coccus hæmolyticus. If the germicidal titers are plotted against the number of carbon atoms in the soap molecule, as in Figs. 1 and 2, it will be seen that the effect of hydroxylation is to shift the curve to the right.

In addition to this general effect, specific effects varying a great deal with the test organisms may be observed. This is best brought out by computing the ratios between the titers of an unsubstituted soap, such as potassium myristate, and the corresponding α-hydroxymyristate. This is done in Table III, which is condensed from Tables I and II. For comparison, the titers for potassium α-bromomyristate from a previous paper (Eggerth, 1929) are included. The last column in Table III shows that the effect of introducing a hydroxyl group (or bromine atom) is not uniform, but varies widely with the species of organism. Table III shows that the hydroxylation of myristic acid greatly increases the germicidal action for *B. typhosus*, *B. pyocyaneus*, *B. melitensis*, *Vibrio choleræ*, and *Staphylococcus aureus* (second value); it diminishes the 2 hour titer for *B. diphtheriæ* (but, as shown in Table I, it greatly increases the 18 hour titer); the titers for the other organisms are practically unchanged. It is to be noticed that the effects of hydroxylation are often quite different from those of bromination (Table III).

With the unsubstituted soaps, the 18 hour titer is in most cases double that of the 2 hour titer (Table II); with the hydroxy soaps, the 18 hour titer is often much greater than that for 2 hours. The most striking example of this is *B. diphtheriæ* with the α-hydroxymyristate, -palmitate, and -stearate, where, at pH 7.5 the 18 hour titer is 256 times as great as the 2 hour titer (Table I).

With one organism, *Staphylococcus aureus*, a very peculiar result was quite consistently obtained. With the α-hydroxymyristate and -palmitate, this germ gave two distinct zones of germicidal action at

TABLE III

The Germicidal Titers of Potassium Myristate, Potassium α-Hydroxymyristate, and Potassium α-Bromomyristate

Test organism	Myristate	α-hydroxy-myristate	α-bromo-myristate	Ratio
Diplococcus pneumoniæ...............	N/2560	N/2560	N/40,960	1:1:16
Streptococcus hæmolyticus.............	N/640	N/320	N/10,240	2:1:32
Staphylococcus aureus.................	N/160	{ N/80 { N/20,480	N/5120	{ 2:1:64 { 1:128:32
B. diphtheriæ........................	N/320	N/80	N/5120	4:1:64
Micrococcus ovalis...................	N/40	N/80	N/2560	1:2:64
Vibrio choleræ.......................	N/160	N/1280	N/1280	1:8:8
B. melitensis........................	N/320	N/1280	N/2560	1:4:8
B. lepisepticus......................	N/2560	N/1280	N/5120	2:1:4
B. pyocyaneus.......................	N/40	N/160	N/20	2:8:1
B. typhosus.........................	0	N/640	N/20	0:16:1

All tests were made at pH 7.5; temperature, 37°C.

pH 7.5 (Table I). Thus, with the hydroxymyristate, concentrations of N/40 and N/80 were germicidal in 2 hours; concentrations of N/160 to N/2560 gave growth on subculture (though often with a much diminished number of colonies); concentrations of N/5120, N/10,240, and N/20,480 were again usually germicidal, though occasionally one to five colonies appeared on the subculture. (A loopful of the control, when diluted and plated out, was shown to give at least 15,000 colonies). Higher dilutions showed no germicidal action. When the time of test was increased to 18 hours, the results were usually still the same, though occasionally all concentrations up to N/20,480 were found to be germicidal. These two zones appeared likewise with the α-

hydroxypalmitate, but not with any other soap. They appeared only at pH 7.5. With *B. diphtheriæ*, a similar phenomenon was occasionally observed with the same soaps, but it appeared so irregularly that it was not indicated in Table I.

The above phenomenon appeared so extraordinary that a number of experiments were undertaken to verify and explain it. These may be summarized as follows:

1. Two other strains of *Staphylococcus aureus*, recently isolated from pus, showed the same phenomenon.

2. Colonies from *Staphylococci* that survived an α-hydroxymyristate concentration of N/320 for 18 hours were fished and tested. They gave practically the same titers as the original stock culture. The resisting forms are, therefore, not mutants.

3. Mixed inoculations of *Staphylococcus aureus* and *Streptococcus hæmolyticus* were tested with α-hydroxymyristate at pH 7.5. Concentrations of N/160 and N/320 gave, on subculture, pure cultures of *Staphylococcus aureus;* concentrations of N/5120, N/10,240, and N/20,480 gave pure cultures of *Streptococcus hæmolyticus*. Mixed inoculations of *Staphylococcus aureus* with *Diplococcus pneumoniæ*, *Vibrio choleræ*, *B. lepiscpticus*, *B. melitensis*, and *B. typhosus* gave similar results.

4. *Staphylococci* were grown on agar and emulsified in sterile buffer in such quantity that the fluid was faintly turbid. Dilutions of α-hydroxymyristate were added as usual. With this large inoculum, organisms could be found with ease in stained films. Subcultures of all dilutions were still positive after 24 hours. After 48 hours, subcultures from soap dilutions of N/160 to N/5120 were negative; films made at the same time showed large numbers of well staining, unagglutinated *Staphylococci*. This experiment rules out agglutination of the organisms by higher dilutions of the soap as a possible cause of the phenomenon.

5. Bacteriostatic tests were made in fluid media. These tests were complicated by the fact that both peptone and meat infusion markedly inhibit the germicidal and growth inhibiting action of the α-hydroxy soaps. In this respect, these soaps differ decidedly from the unsubstituted soaps and sodium oleate (Eggerth, 1927), upon which peptone has no inhibiting action. However, it was found that *Staphylococcus aureus* grows well in a medium having the following composition: N/40 potassium phosphate at pH 7.5; 0.03% Parke Davis peptone, and 0.3% glucose. This small amount of peptone is only slightly inhibitory to these soaps. After 3 days at 37°C. stained films were made from the sediment of each tube, and phenol red was added to determine acid production; these two tests for growth always confirmed one another. The bacteriostatic concentrations of these soaps for *Staphylococcus aureus* were found to be: N/640 for α-hydroxylaurate; N/10,240 for α-hydroxymyristate and -palmitate; N/2560 for α-hydroxystearate. Two other recently isolated *Staphylococci* gave the same results.

6. Instead of subculturing one loopful in the usual way, quantities of 0.1, 0.01, and 0.001 cc. of test fluids were plated out and colony counts made. The results are given in Table IV. This experiment shows that concentrations of the α-hydroxymyristate of N/20,480 or more, rapidly kill off the great majority of the *Staphylococci* present. There are, however, a few survivors in concentrations less than N/80, and the survivors are more numerous in the zone from N/160 to N/1280 than in zone from N/2560 to N/20,480.

It is remarkable that some organisms in a culture can tolerate a concentration of germicide that is 128 times as great as that which

TABLE IV

Colony Counts of Staphylococcus aureus Treated with Potassium α-Hydroxymyristate

Concentration of soap	Number of colonies per cubic centimeter	
	After 2 hrs.	After 18 hrs.
N/80	0	0
N/160	5000	0
N/320	12,000	650
N/640	30,000	3500
N/1280	80,000	5000
N/2560	50	10
N/5120	80	0
N/10,240	30	0
N/20,480	200	250
N/40,960	Innumerable	Innumerable
N/81,920	"	"
Control	"	"

This test was made at pH 7.5; temperature, 37°C.

is lethal to the great bulk of the bacteria in the culture. When the number of survivors is reduced to 100 or less per cubic centimeter a loopful, when cultured, is likely not to give any growth at all, thus giving the second germicidal zone.

The experiment shown in Table IV was repeated several times; the actual colony counts varied a great deal in different experiments, showing that the number of soap resistant organisms in a culture varied greatly. The comparative results, however, remained essentially the same. Attempts were made to determine what factors influenced the proportion of resistant organisms in a culture, but without suc-

TABLE V

The Germicidal Titers of Oleates and Ricinoleates

pH	Oleate		Ricinoleate	
	2 hrs.	18 hrs.	2 hrs.	18 hrs.
Diplococcus pneumoniæ				
6.5	N/327,680	N/327,680	N/20,480	N/81,920
7.5	N/40,960	N/327,680	N/5120	N/20,480
8.5	N/20,480	N/163,840	N/2560	N/20,480
Streptococcus hæmolyticus				
6.5	N/163,840	N/327,680	N/10,240	N/20,480
7.5	N/20,480	N/40,960	N/2560	N/10,240
8.5	N/10,240	N/40,960	N/1280	N/5120
B. diphtheriæ				
6.5	N/81,920	N/163,840	N/10,240	N/20,480
7.5	N/20,480	N/40,960	N/1280	N/2560
8.5	N/10,240	N/20,480	N/320	N/1280
Staphylococcus aureus				
6.5	0	0	N/2560	N/5120
7.5	0	0	N/640	N/1280
8.5	N/40	N/80	N/640	N/1280
Micrococcus ovalis				
6.5	0	0	0	0
7.5	0	N/40	N/160	N/320
8.5	N/40	N/160	N/80	N/80
Vibrio choleræ				
6.5	0	0	N/80	N/320
7.5	0	N/80	N/320	N/320
8.5	0	N/640	N/160	N/320
B. lepisepticus				
6.5	0	0	N/5120	N/20,480
7.5	N/40	N/80	N/2560	N/10,240
8.5	N/640	N/2560	N/1280	N/5120

TABLE V—*Concluded*

pH	Oleate		Ricinoleate	
	2 hrs.	18 hrs.	2 hrs.	18 hrs.
B. melitensis				
6.5	0	0	N/2560	N/5120
7.5	0	0	N/1280	N/1280
8.5	N/80	N/160	N/640	N/640
B. pyocyaneus				
6.5	0	0	0	0
7.5	0	0	0	0
8.5	0	0	0	0
B. typhosus				
6.5	0	0	0	0
7.5	0	0	0	N/40
8.5	0	0	0	N/80

The lowest concentrations tested were N/40.   Temperature, 37°C.

cess; such factors as the age or previous history of the culture, or even the composition of the culture medium, seemed to be without influence.

The effect of the pH upon the germicidal action of the α-hydroxy soaps is, in general, the same as upon other soaps (Eggerth, 1926, 1929).   Two main types of pH effect may be observed.   First, what might be called the "normal pH effect" is illustrated by all of these soaps with *Diplococcus pneumoniæ;* the titer is highest in acid reactions, and diminishes with increasing alkalinity.   Second, with might be called the "reversed pH effect," as in the case of *B. typhosus* with the α-hydroxymyristate; germicidal action is lacking at pH 6.5, but appears and increases with increasing alkalinity.   In some cases a combination of the two effects is evident.   The "reversed pH effect" is to be ascribed to the fact that the soaps are too insoluble at the acid pH to get a germicidal concentration into solution.   In certain cases, the pH effect is of the "reversed" type for the 2 hour period, and of the "normal" type for the 18 hours period, as for *Streptococcus hæmolyticus* with α-hydroxymyristate and -palmitate (Table I).   In the

latter cases it is obvious that enough soap went into solution at pH 6.5 to kill in 18 hours, but not enough to kill in 2 hours.

The effect of introducing a hydroxyl radical into an unsaturated soap is shown in Table V. One is struck by the fact that where the oleate titers are high, those for the ricinoleate are much lower; where the oleate titers are low, the corresponding ones for the ricinoleate are higher (except for *B. pyocyaneus*). The hydroxyl group in this unsaturated soap, therefore diminishes *selective* germicidal action; whereas the hydroxyl group in saturated soaps rather increases selective germicidal action.

It will be noticed that the titers of the oleate for *Streptococcus hæmolyticus* and *B. diphtheriæ* are somewhat higher than those reported for the same two organisms several years ago (Eggerth, 1926 and 1927). This seems to be due to an actual increased susceptibility of these two cultures, as several samples of Kahlbaum's oleic acid and sodium oleate were tried, and all gave the same high titers.

The Selective Germicidal Action of Soaps

It has been known for some time that the unsaturated soaps, such as the oleates, will destroy certain organisms such as *Diplococcus pneumoniæ* and *Streptococcus* in high dilutions, and yet have very little toxicity for other species, as *Staphylococcus* (Avery, 1918). Walker (1924) has more recently shown that the saturated soaps, especially the laurate, likewise show considerable selective germicidal action. The writer has tested ten organisms against three complete series of soaps, and has been repeatedly impressed by the fact that all of these soaps show selective germicidal action, though no two of them in exactly the same way. That being the case, it should be possible, by selecting the right soap, to kill at will any one of a mixture of organisms. This was actually done in a number of instances. Thus, in a mixture of *Streptococcus hæmolyticus, Staphylococcus aureus, B. diphtheriæ*, and *B. typhosus*, the soap potassium α-bromostearate in concentrations of N/40,960 and N/81,920 killed only the *Streptococcus* in 2 hours at pH 7.5; the α-hydroxymyristate in concentrations of N/5120, N/10,240, and N/20,480 killed only the *Staphylococcus* at pH 7.5; the α-hydroxystearate in concentrations of N/2560 to N/40,960 killed only *B. diphtheriæ* in 18 hours at pH 7.5; while the α-hydroxy-

myristate at pH 8.5 killed only *B. typhosus* in a concentration of N/1280. Other combinations of four organisms could not be arranged so successfully, but numerous two-organism combinations were tested with the desired results. Thus N/5120 and N/10,240 potassium laurate kills *B. lepisepticus* at pH 7.5 in 18 hours, but not *Diplococcus pneumoniæ;* whereas potassium oleate kills the latter in high dilutions, but not the former. In a mixture of *Streptococcus* and *Vibrio choleræ*, oleate or α-bromostearate destroys the *Streptococcus;* α-hydroxymyristate, the *Vibrio*. In a mixture of *Micrococcus ovalis* and *B. pyocyaneus*, α-bromostearate kills the former; α-hydroxymyristate at pH 8.5, the latter. Other similar combinations are possible.

As soon as data on other soaps and other organisms is obtained, it is likely that even more striking examples of selective germicidal action will be encountered. The possibilities are interesting.

SUMMARY AND CONCLUSIONS

1. The α-hydroxy soaps exhibit a high germicidal action toward certain organisms. As with other soaps, the germicidal action increases with molecular weight to a maximum, then diminishes. The pH effects the germicidal action as it does other soaps.

2. Certain α-hydroxy soaps give two distinct germicidal zones with *Staphylococcus aureus*.

3. The effect of the hydroxyl group in saturated soaps is to increase selective germicidal action; the effect of the hydroxyl group in an unsaturated soap is to diminish it.

4. The soaps offer a means of separating mixtures of organisms by selective germicidal action.

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PROPERTIES OF THE CAUSATIVE AGENT OF A CHICKEN TUMOR

I. The Specific Fixation by Tissues of Susceptible Animals[*]

By F. DURAN-REYNALS, M.D., and JAMES B. MURPHY, M.D.

The discovery that a group of malignant tumors of the fowl could be transmitted by a cell-free extract or a desiccate of the tumor has lead to a great diversity of opinion, not only as to the classification of the tumor group, but also as to the nature of the agents possessing the tumor producing property. By many these neoplasms are classed among the virus diseases. However there are certain biological properties of these tumors and some experimental data which are difficult to reconcile with the theory that the agents are similar to those causing the virus type of infectious disease process.

Various attempts have been made to devise means of distinguishing between the filter-passing virus group, presumably living organisms, and the active substances, products of living cells, the enzyme-like group of agents. But as far as the chicken tumors are concerned, it cannot be considered that any of the earlier studies offered very definite information as to the character of the causative agents.

Certain biological agents such as bacteriophage, enzymes, toxins and antibodies, all more or less selective in the cells or substances acted upon, are first adsorbed or fixed, and sometimes apparently inactivated by the specific substratum, while non-specific cells or substances are without effect on the agents. For example, tetanus toxin, which has a selective action on the nervous system, is neutralized *in vitro* by nervous tissue from susceptible animals, while kidney, spleen and other organs from the same animals have little or no effect (1). Furthermore, the brain tissue of animals non-susceptible to tetanus toxin has practically no neutralizing effect *in vitro* on the poison (2) (3).

There are some indications that the chicken tumor agent may be bound by tissues under certain conditions.   Pentimalli (4) observed that the activity of a chicken tumor filtrate was reduced by contact with chick embryo pulp, and also by the repair tissue from a healing wound in chickens, but in a lesser degree.   Leucocytes had no activity in this respect.   Deelman reports a similar observation (5).

The present work has to do with a systematic study of the action of tissues of susceptible fowls on the chicken tumor agent as compared with tissues of non-susceptible animals.   A preliminary note on the subject has already been published (6).

Experimenal Method

The tumor filtrates were prepared by grinding about 5 gms. of tumor tissue with sand and then adding 100 cc. of Ringer's solution.   After a thorough shaking the suspension was centrifuged to remove the sand and larger particles, and the supernatant fluid passed through a Berkefeld V filter.   The tissues to be tested were ground to a fine pulp in a mortar with the addition of Ringer's solution in the ratio of about 2 gms. of tissue to 1 cc. of fluid.   A measured amount of the resulting pulp was placed in a centrifuge tube and a measured amount of the Berkefeld filtrate of the tumor was added and mixed thoroughly.   After a period of contact, either at room temperature or in the incubator, the mixture was centrifuged and the activity of the supernatant fluid was tested by intradermal injections in normal chickens.   The activity of each filtrate was tested by the injection of an untreated sample in a similar manner.

Preliminary Experiment:—Five preliminary experiments were undertaken on a small number of animals in order to gain some idea as to the quantitative relationships and the degree of specificity of the reaction.   As the technique was almost identical in the several experiments, the results are presented in tabulated form in Table I.

From these preliminary tests it would seem that the muscle of susceptible chickens definitely reduces the amount of activity of the tumor agent in the filtrate.   On the other hand, the brain and liver from the same chickens, and muscle, brain and liver from rabbits have no detectable effect on the potency of the tumor agent.

Fixation and Inactivation Experiments with Muscle from Chicken, Rabbit and Pigeon:—The next step was to duplicate the above experiments on an animal more closely related to the chicken, namely the pigeon, using larger amounts of tumor filtrate.

Eight cc. of the muscle pulps were mixed with 4 cc. of fresh

TABLE I

	Mixtures	Time of contact	Resultant tumor from supernat. fluid
Exp. 1.	9 cc. chicken muscle pulp + 1 cc. tumor filtrate	3 hrs. at 31°	+
	9 cc. rabbit " + 1 cc. " "		++++
Exp. 2.	8 cc. chicken " + 3 cc. " "	1 hr. at 37°	++
	8 cc. rabbit " + 3 cc. " "		++++
	3 cc. chicken brain + 1 cc. " "		++++
	3 cc. rabbit " + 1 cc. " "		++++
Exp. 3	8 cc. chicken muscle + 2 cc. " "	3 hrs. at 37°	−
	8 cc. rabbit " + 2 cc. " "		++++
	8 cc. chicken liver + 2 cc. " "	—	++++
	8 cc. rabbit " + 2 cc. " "		++++
	3 cc. chicken brain + 0.8 cc. " "	1 hr. at room temp.	++++
	3 cc. rabbit " + 0.8 cc. " "		++++
Exp. 4	8 cc. chicken muscle + 2 cc. " "	3 hrs. at 37°	−
	8 cc. rabbit " + 2 cc. " "	1 hr. at room temp.	++
Exp. 5	8 cc. chicken " + 2 cc. " "	5 hours	−
	8 cc. rabbit " + 2 cc. " "		++++

tumor filtrate and allowed to remain in contact at room temperature for 3 hours for Experiment 12 and 14, and 4 to 6 hours for all the others. One cc. of the supernatant fluids was injected intracutaneously, and a similar amount of the tissue pulp in the breast muscle of normal chickens. The results are given in Table II.

TABLE II

Exp. no.	Tumors from supernatant fluids 1 cc.		Tumors from pulps 1 cc.		Tumors from filtrate alone	
	Chicken muscle	Rabbit muscle	Chicken muscle	Rabbit muscle		
6	− −	++++ ++++	−	++++	0.5 cc. ++++	1 cc. ++++
7	++ +++ ++	++ ++++ +++	+++ +++ +	+ +++ ++	0.5 cc. ++ 5 cc. ++++	1 cc. +++ 10 cc. ++++
		Pigeon muscle		Pigeon muscle		
8	−	−	−	+++	1 cc. +++	2 cc. ++++
9	− + −	− +++ −	− −	− −	0.5 cc. −	1 cc. −
10	++ +	+++ ++++	+++ +++	+++ ++++	0.5 cc. +++ 2 cc. +++	1 cc. +++ 4 cc. ++++
11	− +	++++ ++++	+++	+++	1 cc. +++ 5 cc. ++++	2 cc. ++++ 5 cc. ++++
12	++ −	+++ −	++++ −	+++ −	1 cc. ++++ 2 cc. −	0.5 cc. +++ 4 cc. −
13	+++ ++++ ++++	+++ +++ ++++	++ ++++	+++ ++	0.5 cc. ++++	1 cc. ++++
14	++ ++++ ++++	++ +++ ++++	++++ ++	++++ ++	1 cc. ++	5 cc. +++

It will be noted that more tumor filtrate was used with the tissue pulp in this group of experiments than in the first group. Out of the nine experiments five gave evidence of reduction in activity of the filtrate after contact with chicken muscle pulp, while one was negative, and three showed no evidence of reduction in activity of the filtrate by chicken muscle contact. With the exception of No. 6, and probably No. 8, the injection of the pulps, both from the chicken muscle and the controls, showed them to be of about equal potency in the production of tumors.

The fact that a proportion of these experiments failed to show any marked reduction in the activity of the filtrate in contact with muscle pulp from susceptible animals suggested the possibility that too active a filtrate had been used. To test this several dilutions of the filtrate were utilized in the next group of experiments. For the results see Table III.

TABLE III

Exp. no.	Tumors from supernatant fluids 1 cc.			Tumors from pulps 1 cc.	
	Chicken muscle	Rabbit muscle		Chicken muscle	Rabbit muscle
15	++ ++	+++ +++	Pure Filtrate	++++ ++	++++ +++
	+ +	+++ ++	1:1 Filtrate	++ +++	++++ ++++
	− ?	++ ?	1:2 Filtrate	+++ ++	++++ ++++
	Chicken muscle	Pigeon muscle		Chicken muscle	Pigeon muscle
16	− −	++ ++++	Pure Filtrate	?	?
	+ −	++ +++	1:1 Filtrate	++++	++++
	+ −	+++ +++	1:2 Filtrate	++	+++
	+ +	+++ ++	1:3 Filtrate	+	+++

In addition to the animals included in Table III, each of the two sets of supernatant fluids of experiments 15 and 16 from the three dilutions of filtrate were tested on the same chicken, 0.2 cc. of each being injected intradermally.   The results are summarized in Table IV.

TABLE IV

Tumors from Supernatant Fluids

Exp. no.		Chicken muscle	Rabbit muscle
15	Pure Filtrate	++	+++·
	1:1 Filtrate	++	++
	1:2 Filtrate	+	+++
16	Pure Filtrate	(4) −	(8) ++++
	1:1 Filtrate	(3) −	(7) +++
	1:2 Filtrate	(2) −	(6) ++
	1:3 Filtrate	(1) +	(5) ++
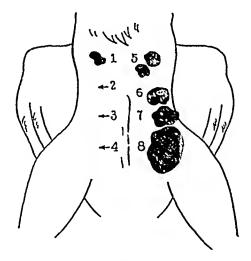
FIG. 1

Fig. 1 gives a schematic representation of the chicken used in Experiment 16, showing the relative sizes of the tumors 16 days after intradermal inoculation.

It appears from these experiments that the amount of tumor agent fixed or inactivated by chicken muscle has a definite quantitative limit.

The difference in activity between the muscle of susceptible animals and of resistant ones is more clearly demonstrated when the filtrates are diluted. The results of the injection of the muscle pulps would suggest that the fixation power of the chicken muscle is stronger than its inactivating power.

Fixation and Inactivation by Desiccated Muscle:—It was desirable to know whether the properties found in the chicken muscle were properties of the living cell in the ground tissue or were reactions between the tumor agent and the tissue constituents. The following experiment shows that dried chicken muscle is able to fix and inactivate the chicken tumor agent almost if not quite as actively as the fresh pulp.

Experiment 17:—The dry muscle used in this experiment was from the same fowl as that used in the fresh state in Experiment 6, when complete inactivation of the filtrate had taken place. The tissues used were dried in a vacuum over sulphuric acid, and then ground to a coarse powder. One gram of each muscle desiccate was mixed with 2.5 gms. of Ringer's solution and after the addition of 2 cc. of fresh tumor filtrate they were kept for two hours at 36°C. and three hours at room temperature. After centrifuging the mixture 1 cc. of each was injected intradermally in normal chickens, and the pulps were given in the same amounts intramuscularly. The activity of the filtrate alone was tested by the injection of 5 cc. intramuscularly. The results are given in Table V.

TABLE V

Tumors from supernatant fluid 1 cc.		Tumors from pulps 1 cc.		Tumors of control from 5 cc. of filtrate alone	
Chicken muscle	Rabbit muscle	Chicken muscle	Rabbit muscle		
+	++++	+	++++	++++	++++

The experiment shows quite clearly the inactivation as well as the fixation of the agent by the dry chicken muscle.

Fixation and Inactivation Experiments with Brain and Liver from Chicken, Rabbit and Pigeon:—The same relative proportions of the organ pulps and fresh tumor filtrate were used as those used in the foregoing experiments with muscles. As indicated by the experiment number, these tests were performed simultaneously with the muscle experiments, the length of contact being the same. The results are given in Tables VI and VII.

TABLE VI
Brain

Exp. no.	Tumors from supernatant fluid 1 cc.		Tumors from pulps 1 cc.		Control tumors from filtrate alone	
	Chicken brain	Rabbit brain	Chicken brain	Rabbit brain	Right side	Left side
7	++ ++	++ ++	++ ++	++ ++	0.5 cc. ++	1 cc. +++
18	+++ ++	+++ ++	+++	+++	5 cc. −	10 cc. −
		Pigeon brain		Pigeon brain		
10	+++	+++	++++ ++++	++++ ++++	0.5 cc. +++ 2 cc. +++	1 cc. +++ 4 cc. ++++
12	− −	− −	+++	+++	1 cc. ++++ 2 cc. −	0.5 cc. +++ 4 cc. −

TABLE VII
Liver

Exp. no.	Tumors from supernatant fluids 1 cc.		Tumors from pulps 1 cc.		Control tumors from filtrate alone	
	Chicken liver	Rabbit liver	Chicken liver	Rabbit liver	Right side	Left side
7	++ + ++	++ + ++	+++ +++ ++	+++ +++ +	0.5 cc. ++ 5 cc. ++++	1 cc. +++ 10 cc. ++++ ,
18	++ +++ ++	++ +++ ++	− −	− −	0.5 cc. − 5 cc. −	1 cc. − 10 cc. −
		Pigeon liver		Pigeon liver		
13	+++ +++ +++	+ +++ +++	++++ ++++	++ ++	0.5 cc. ++++	1 cc. ++++
14	++++ ++ +	++++ ++++ +	+++ +++	+++ ++	0.5 cc. ++	1 cc. ++++

It will be noticed in Experiments 7, 13 and 14 that the simultaneous experiments dealing with muscle gave negative results, due to the excessive activity of the filtrate. Therefore, as far as chicken liver and brain are concerned, these experiments show at least that they are not endowed with a stronger inactivating power than muscle. Experiments 10 and 12 show pronounced fixation with muscle so that a proper control exists for these two experiments, and as the filtrate used in Experiment 19 was weak it is possible that any fixation would have been detected in this experiment.

It seems, therefore, that not only do the liver and brain of rabbits and pigeons fail to show any fixating or inactivating properties for the chicken tumor agent, but that is true as well of the same organs of the chicken.

In addition to the experiments above, two other tissue were tested for the fixating power on the chicken tumor agent, namely, a mouse sarcoma and a non-filterable chicken tumor. In neither tissue was there any indication of fixation while two of the four control tests with chicken muscle showed complete fixation of the agent.

Attempts to Release the Agent from the Inactive Muscle-Filtrate Mixtures:—As noted by Marie and Tiffeneau (7), desiccation of the inactive mixture of brain and tetanus toxin releases the toxin. The technique was used in an attempt to release the tumor agent from the susceptible muscle.

Experiment 19:—The pulps from Experiment 11 were desiccated in a vacuum over sulphuric acid and four days later injected in an amount equivalent to 4 cc. of the fresh pulp.

The results obtained 15 and 30 days after the injection are given in Table VIII.

TABLE VIII

	Tumor from chicken musc.	Tumor from pigeon musc.		Tumor from chicken musc.	Tumor from pigeon musc.
15 days	−	+++	30 days	++	++++

It seems that the agent already fixed and inactivated is not released by desiccation. In fact, in this particular experiment, the injection

of dried pulps showed a greater contrast between the actions of the two muscle mixtures than did the fresh mixture as shown in Experiment 11.

General Comparison of Results with Muscle from Susceptible and Non-susceptible Animals:—If all the tests with muscle of susceptible chickens used in the above experiments be compared with the results with pigeon and rabbit muscle, striking contrast between the groups will be noted. (Table IX.) In the 47 tests with chicken muscle 76% showed unmistakable evidence of some fixation of the tumor agent, and in 34% this was complete. On the other hand, with the 43 tests with muscle from non-susceptible animals there were no instances of fixation. The figures for inactivation, while not based on so many tests show a strong contrast between chicken muscle on the one hand and the rabbit and pigeon muscle on the other.

TABLE IX

Results of Injection of Supernatant Fluids

Muscle from	Number chickens inoculated	Total fixation	Partial fixation	No fixation	Percentage tot. and part fixation	Percentage total fixation
Rabbit.....................	19	0	0	19	0.0	0.0
Pigeon.....................	24	0	0	24	0.0	0.0
Chicken....................	47	16	20	11	76.7	34.4

Results of Injection of Pulps

Muscle from	Number chickens inoculated	Total inactiv.	Partial inactiv.	No inactiv.	Percentage tot. and part inactiv.	Percentage total inactivation
Rabbit.....................	12	0	1	11	8.3	0.0
Pigeon.....................	12	0	1	11	8.3	0.0
Chicken	24	2	9	13	45.8	8.3

DISCUSSION

The evidence developed in this study seems to indicate that the agent of Chicken Tumor I is bound *in vitro* by muscle tissue from susceptible fowls while the muscle and the tissues from non-susceptible animals such as rabbit and pigeon are devoid of any such action. As

far as the present observation goes, even such non-mesenchymatous organs as the brain and liver of the susceptible fowl show no affinity for the tumor agent *in vitro*.   It is of interest in this connection to note the early observations of Murphy and Rous (8) who showed that when tumor filtrate was injected into the chick embryo, tumors developed only in the mesodermal layers of the embryonic membranes.

The question naturally arises as to whether it is solely the muscle cell, the sarcolemma or both which are responsible for the binding action of the pulp.   Although this point does not modify the essential nature of the phenomenon, the supposition that a transformation of the differentiated muscle cell under the action of the agent into a malignant cell does not seem theoretically unreasonable.

The interaction between the muscle of susceptible fowls and the tumor agent resembles in its specificity the binding of the antibodies by antigens, bacteriophage by the sensitive bacteria and the enzyme by the specific substratum.   The muscle tumor agent combination seems to be rather stronger than some of the examples quoted, as desiccation does not release the activity.   The delicacy of the tumor agent prevents more extensive attempts to dissociate the combination with muscles.   It is true that certain viruses also have a high degree of specificity in animals and plants and share with the tumor agent the property of requiring living matter for their multiplication.   However, it has been demonstrated (9) that at least one typical virus, the vaccine virus, is not bound or inactivated by contact with sensitive tissue from susceptible animals.   This is possibly a fundamental difference between the behavior of the chicken tumor agent and the filterable agents of the virus group.

CONCLUSIONS

Ground muscle from susceptible chickens fixes in vitro in a proportion of instances the agent of the filterable Chicken Tumor I, and in a lesser degree inactivates it, whereas the muscle from resistant animals such as rabbit and pigeon, is without effect.   It is shown that the power of fixation of the chicken muscle is far greater than its inactivating properties.

Brain and liver from chicken, rabbit and pigeon seem devoid of any action on the agent.

The desiccated chicken muscle tissue shares the properties of the fresh organ; and the process of desiccation does not release the agent from the inactive or slightly active mixture of fresh muscle and filtrate.

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THE EFFECT OF EXTRACTS OF CERTAIN ORGANS FROM NORMAL AND IMMUNIZED ANIMALS ON THE INFECTING POWER OF VACCINE VIRUS

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PLATES 14 AND 15

In a previous paper (1) we reported that when the agent of Chicken Tumor I was brought in contact with finely ground muscle from susceptible animals it was bound so that both the supernatant fluid and the muscle tissue became partially or completely inactive when injected into chickens. The muscle of non-susceptible animals such as pigeon and rabbit, or even organs such as liver, brain and kidney of susceptible chickens do not affect the activity of the agent.

The present study was undertaken in order to determine whether a similar effect could be demonstrated for a typical representative of the group of the so-called filter-passing viruses, such as vaccine virus. As laboratory animals are more or less susceptible to vaccine virus, for the refractive group it has been necessary to use artificially immunized rabbits, although it is realized that subsequent work may show that the comparison is unjustified.[1]

The fact that tissues of immunized animals inactivate the virus *in vitro* has already been shown. Thus Levaditi and Nicolau (4) report that vaccine virus and herpes virus were "destroyed" by the brain tissue from immunized animals. Tessier, Gastinel and Reilly (5) using a small number of animals confirmed the above result with herpes virus and further determined that the brain and serum from naturally immune rabbits and non-susceptible dogs are devoid of action on the virus.

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[1] A preliminary note on the subject has already been published and some of its points have been duplicated on the staphylococcus (2), (3).

Methods and Materials

Vaccine Virus.—Three different strains of virus have been used, namely, the Noguchi testicular vaccine virus, the Levaditi neurovaccine, and the cow glycerinized dermovirus of the Department of Health, City of New York.[2]

The specimen of testicular virus was prepared by injecting 0.5 cc. of vaccine emulsion mixed with an equal amount of Ringer's solution into both testicles of a rabbit. 5 days later, when the resultant orchitis was at its height, the animal was killed, and the testicles removed aseptically. They were ground thoroughly with sand together with 25 cc. of glycerol and 25 cc. of Ringer's solution. This emulsion was distributed in tubes, covered with a layer of sterile vaseline, and kept in the ice-box.

The specimen of neurovirus was prepared by injecting about 0.2 cc. of a heavy suspension of infected brain tissue into the brain of a rabbit. After 5 days the brain was removed aseptically and treated in the same manner as the testicular virus. Dilutions, usually at 1:50, were made immediately before each experiment with Ringer's solution.

The animals were carefully shaved, so as to avoid any injury of the skin, and generally a test injection was made intracutaneously on one side, the opposite side being used as control in order to avoid the disturbing fact of the individual differences in susceptibility.

Inactivation of Testicular Virus by Immune Brain Extract.—As mentioned above, Levaditi has shown that an extract of the brain of an immune animal neutralized the neurovaccine. This experiment has been repeated, substituting Noguchi's testicular stain for the neurovirus.

The brains of two rabbits immune to the testicular virus were used, controlled by the brains from two normal rabbits. A half of each brain was ground and mixed with 2 to 3 cc. of a 1:10 virus dilution. The mixture was kept for 5 hours at room temperature in one instance and in the other for 3 hours at 37° and then overnight in the ice-box. The mixtures were then centrifuged and the supernatant fluids and pulps injected separately into normal rabbits. The results are shown in Table I.

TABLE I

Exper.	Lesions produced by supernat. fluids		Lesions produced by pulps	
	Immune brain	Normal brain	Immune brain	Normal brain
I	0.5 cc. ±	+	0.1 cc. −	+
	0.5 cc. ±	+	0.1 cc. ±	±
	0.5 cc. −	+	0.1 cc. ++	++++
II	0.5 cc. −	±	0.1 cc. +	+
	0.5 cc. −	−	0.1 cc. +	+

Inactivation of Testicular Virus by Immune Testicle Tissue.—The same experiment was repeated using testicle instead of brain tissues.

Immune rabbits from 1 to 3 months after the immunizing injection of testicle or neurovirus were used. The testicles were removed and ground without sand, and measured amounts of the resultant pulps were mixed with equal volumes of 1:50 testicle virus dilution. The length of contact was 3 hours at room temperature in one instance and from 1 to 3 hours at room temperature and overnight in the ice-box for the rest of the experiments. The mixtures were then centrifuged and the supernatant fluids injected into normal rabbits. The same procedures were carried out with normal testicles in the control experiments.

The results are summarized in Table II.

TABLE II

Exp. No.	Lesions by supernat. fluids from:		Lesion by pulps from:	
	Immune testicle	Normal testicle	Immune	Normal
3	0.2 cc. — 0.5 cc. ±	+++ ++++	0.2 cc. —	+
4	0.2 cc. ± 0.5 cc. ±	+++ +++	0.1 cc. — 0.1 cc. —	+++ +++
5	0.2 cc. — Scarification —	+++ ++	Scarification —	+
6	0.5 cc. —	++	No test	No test
7	0.2 cc. +	++	0.1 cc. +	++
8	0.3 cc. —	++	0.1 cc. —	++
9	0.5 cc. —	+++	0.1 cc. —	++
10	0.5 cc. —	+++	0.2 cc. —	±
11	0.5 cc. — 0.5 cc. —	+++ +++	0.2 cc. + 0.2 cc. +	+++ ++

It is evident from these tests that the brain and the testicle from immune animals inactivate vaccine virus, the latter tissue being the

more active.   The inactivation takes place quickly, as a contact of 3 hours reduced the activity of the virus dilution as completely as a longer contact.[3]

Perfusion Experiments.—It is known that the serum from an immune animal exhibits so-called viricidal action on vaccine virus. Hence the results obtained so far might be ascribed to the inactivating power of the blood contained in the tissue and not the tissues themselves.   To check this point the following experiments were performed.

A 3 months immune rabbit was perfused under anesthesia with Ringer's solution and sodium citrate through the abdominal aorta and the vena cava until the testicles became perfectly white.   The same procedure was carried out on a normal rabbit and the above experiments were repeated with this material.   The mixture of testicle and virus was kept at room temperature for 3 hours and then overnight in the ice-box.   A similar experiment was carried out with neurovirus.   The results are given in Table III.

TABLE III

Virus	Lesion by supernatant fluids		Lesion by pulps	
	Immune testicle	Normal testicle	Immune testicle	Normal testicle
Testicle	1 cc. −	++++	0.1 cc. ±	++
Neuro-	1 cc. +	Very extensive lesion	0.1 cc. +	++++

It appears that the lesions from immune animals possess an inactivating power as well as the serum.

Speed of Reaction between Vaccine Virus and Immune Organs.—A

[3] The question of whether or not the testicle tissue from suscepticle animals fixes the virus was answered by a set of experiments in which normal rabbits were injected with testicle emulsions immediately after the mixtures were made and again after some hours of contact.   As controls the same amount of virus dilutions as those contained in the mixtures was also injected at the same time. As there was no difference in the extent of the lesions produced it may be concluded that there was no fixation of the virus by normal testicle tissue.   That there was a mechanical retention or adsorption of the virus by the tissue was shown by the fact that washing of the tissue after contact did not materially reduce the activity of the material when injected into animals.

further attempt was made to determine the speed of reaction and its further progress during the time of contact.

3 cc. of ground testicle tissue from a 35 day immune rabbit was mixed with an equal volume of a 1:50 testicular vaccine dilution. Mixtures were immediately centrifuged and a sample of the supernatant fluids injected intrademally into a normal rabbit. The remaining fluid and tissue were mixed and allowed to remain in contact for 5 hours at room temperature and then for 17 hours in the ice-box. The mixture was again centrifuged and another sample of the supernatant fluid was tested on the same rabbit. A similar experiment was carried out with neuro-virus. The results are given in Table IV.

TABLE IV

Virus	Lesions produced by supernatant fluids immediately after mixing		Lesion by supernatant fluids and pulps after 22 hours of contact	
	Immune testicle	Normal testicle	Immune testicle	Normal testicle
Testicular	0.5 cc. −	+++	0.25 cc. + 0.50 cc. + 0.10 cc. ±	+++ ++++ +++
Neuro-	0.5 cc. +	Very large lesion	0.25 cc. + 0.50 cc. +	++++ ++++

These tests indicate that the inactivation of the virus by the testicle from immune animals is accomplished very quickly and is not notably increased by further contact.

The possibility that the virus was inactivated but not destroyed by the contact with tissues from immune animals was tested. The mixtures subjected to tryptic digestion, desiccation or treatment with solutions of different pH failed to release the virus in active form.

Enhancement of Neurovirus by Normal Testicular Extract.—It will be noted that in the above experiment the neurovirus was inactivated by the testicular virus. On the other hand, the normal testicular extracts, which are devoid of appreciable effect on the testicular strain, gave evidence of an enormous augmentation in the skin lesions pro-

duced by the neurovirus.   This observation was confirmed by the experiments which are summarized in Table V.

TABLE V

Lesions produced by the supernatant fluid from:		Lesions produced by the pulps from:	
Immune testicle	Normal testicle	Immune testicle	Normal testicle
1. 0.2 cc. +	Very large lesion	0.2 cc. +	Very large lesion
2. 0.2 cc. ++	Very large lesion	0.2 cc. +	++++
3. 0.2 cc. +	Very large lesion	0.2 cc. +	++

The lesions were much more hemorrhagic and necrotic and spread over a much greater area than those produced by the virus alone.   The two rabbits injected with the supernatant fluid from the mixture of normal testicular extract plus neurovirus became very sick, lost considerable weight, and died 6 and 7 days respectively after the injection. One of the rabbits injected with the pulp showed also a large lesion and became sick but eventually recovered.   The other rabbits showed the usual vaccinal eruption.

Detailed Study of the Enhancement of the Neurovaccine Virus by Testicle Extracts.—The nature of the lesions obtained in the foregoing set of experiments warranted a further and careful study of the power shown by normal testicle extracts in enhancing to such a high degree the infecting power of the Levaditi neurovirus.

The technique at first was the same as in the foregoing experiments, and pulps as well as supernatant fluids were injected.   Later on the procedure was simplified by grinding the testicle together with its volume of Ringer's solution and centrifuging immediately.   Only the cloudy supernatant fluid was used.   About 0.5 cc. of this was mixed with 0.25 cc. of a 1:50 dilution of infected tissue emulsion immediately before the injection.

With the supernatant fluid the enhancement has been observed in practically all of the 80 tests carried out.   In 2 or 3 instances where the enhancement was doubtful or negative, the effect was traced to the use of a feeble virus or atrophic testicles.   The injection of the pulps in the amounts used did not give constant results, as in 5 out of 11 instances there was no enhancement, and in 2 cases only doubtful enhancement.

Only in a few instances did the virus-testicle mixture give an earlier lesion than the virus alone. Generally on the 2nd or 3rd day evidence of the infection became detectable on both sides, but the lesion was very much less extensive in the control side. The lesions produced by the neurovirus alone generally reached their maximum spread by the 4th or 5th day and presented the usual picture of a more or less hemorrhagic localized eruption. On the other hand the lesions produced by the virus plus testicle extract generally continued to spread till the 6th or 7th day, by which time they had extended throughout the flank and even over the abdomen. The skin appeared thick and very red or violaceous in color, and it was often covered with blisters. Areas of necrosis often appeared in the middle of the lesion and sometimes the whole of the skin area was necrosed. There was generally a marked oedema in the neighbouring regions.

General symptoms in the rabbits injected intradermally with the testicle-neurovirus mixture became pronounced. The temperature reached 105°F. and sometimes 106° and 107°. In most cases hypothermia followed in the days immediately before death. There was considerable loss in weight—sometimes more than 600 gm.—and most of the animals developed severe diarrhea and signs of pulmonary disease. Conjunctivitis was observed in a few instances.

Death occurred in about 25 per cent of cases. The most striking findings at postmortem examination were a double hemorrhagic lobar pneumonia with sometimes widespread pulmonary abscesses and severe glomerulo-nephritis. Lymph nodes in the vicinity of the lesion were enlarged and congested and the testicles were sometimes congested. The details of the histological lesions of this generalized vaccinal infection will be reported in a later paper. Typical alterations were found in the ovaries, tesicles, suprarenals, lungs, etc., and vaccine virus was easily recovered from these and other organs independently of its presence in the blood.

In other animals purposely killed at the height of the disease, more or less pronounced lesions were also found in the lungs. The general clinical and histo-pathological picture of disease caused by the vaccine virus plus testicle extract is that of the usual infection by the virus alone, but extraordinarily enhanced.

The Influence of Other Organ Extracts on Neurovirus Infection.—It was obviously interesting to know how organs other than testicle behave when injected along with neurovirus.

The same technique was employed, the supernatant fluids only being used. The mixture of virus and tissue extracts or serum was made immediately before injection, in the ratio of 0.25 cc. of a 1:50 dilution of the virus emulsion to 0.50 cc. of the organ or tissue extract.

The results are summarized in Table VI.

TABLE VI

Organ extract injected with neurovirus	No. of tests	No. enhanced	No. un-modified	No. decreased	No. sup-pressed
Testicle..............................	80	78	2	0	0
Kidney..............................	6	6	0	0	0
Skin.................................	2	2	0	0	0
Suprarenal..........................	4	0	4	0	0
Blood (whole).......................	3	0	1	2	0
Serum...............................	10	0	5	5	0
Bone marrow........................	8	0	5	3	0
Lymph nodes........................	2	0	2	0	0
Spleen..............................	24	0	8	14	2

In addition to the above, one test each was made with several other tissues.   The indications from these are that liver, brain and placenta enhance the infection, while muscle, retina and the whole embryo are without effect.

From the study of the results we are able to classify the organs or tissues as regards their influence on vaccinal infection as follows: (1) organs that always enhance, such as testicle, kidney, etc.; (2) organs which neither enhance nor interfere, as, probably, muscle, suprarenal, etc.; (3) organs which never enhance and very often interfere with and even suppress the infecting power of the neurovirus, such as spleen, blood, etc.

Among the organs endowed with an enhancing power the testicle is by far the strongest, whereas the spleen seems most active among the organs which interfere with the infection.   It is interesting to note that the inhibiting power of the spleen is quickly lost by dilution, the pulps being the most effective, whereas the enhancing power of the testicle, as will be seen later, is unaffected by high dilutions.

Enhancement of the Dermovaccine Virus by Testicle Extracts.—In view of the fact that the enhancement of the vaccine virus in the skin was positive with the neuro- and negative with the Noguchi testicular virus, it was desirable to determine whether the testicular extract was capable of enhancing the activity of the usual cow dermovirus.

Rabbit testicle extract was prepared in the usual way, and 0.5 cc. was injected intradermally together with the entire content of a tube of dermovirus (a human dose) into the left side of two rabbits.   The right side was injected with the virus

alone with Ringer's solution.   The left side showed lesions 5 times larger than the right side, but these lesions were milder than those caused by the neurovirus or the testicle virus.

Enhancement of the Noguchi Testicle Virus.—That the feebler activity of the Noguchi testicle virus is not responsible for its lack of activation by testicle extract is clearly shown by the foregoing experiment where a still feebler virus, the cow virus, is definitely enhanced by the same extract.

Kidney extracts proved to be effective in one experiment in enhancing slightly but definitely the testicle virus infection in the skin.   In another experiment testicle virus was injected into the brain of a rabbit which was killed 5 days afterwards, and this brain, used as neurovirus, proved to be definitely enhanced by testicle extract.   The lesions obtained were the usual mild lesions produced by the testicle virus.   On the other hand, the neurovirus after growth in the testicle lost none of its virulence and was still actively enhanced by testicle extracts when injected intradermally.   It is therefore obvious that enhancement of testicle virus by organ extracts is possible under certain conditions.

Enhancement of Neuro- and Testicle Virus by Testicle and Kidney Extracts when Injected into the Testicles.—These experiments were performed in order to determine the effect of the enhancement in organs other than skin, namely, testicles.

The general procedure in preparing the material does not differ from that used in the skin experiments.   The right testicle was used for injections and the left used for comparison.   The results of 4 experiments on 16 rabbits are summarized in Table VII.

TABLE VII

Lesion produced by:				Lesion produced by	
	Testicle virus plus organ ext.	Testicle virus plus Ringer's		Neurovirus plus organ ext.	Neurovirus plus Ringer's
Testicle.......	+	++	Testicle.......	+++	+++
Kidney..	+++	−[a]	Testicle......	++++	+++
Testicle......	+++		Testicle......	++++	
Kidney.......	+++	−	Kidney.......	±	+
Kidney.......	+		Kidney.......	++++	

[a] This minus sign does not show complete absence of orchitis but simply the complete absence of any gross inflammation.   That infection had taken place is shown by the fact that an active virus may be recovered.

From the study of this table we can draw the conclusions that, in spite of some irregularity of both neuro- and testicle virus in giving rise to definite orchitis in rabbits, both can be enhanced by either testicle or kidney extract when injected into rabbit testicles.

Experiments on the Nature of the Enhancement.—The enhancement is not species specific, as the tests show that rabbits injected with neurovirus plus rat or guinea pig testicle extract, prepared and used as the rabbit extract, exhibit typical enhanced lesions. All animals injected became sick and some died on about the 8th day, with the usual general signs especially in lung and kidney. The age of the animal from which the organs for the extracts are taken, as well as the age of the injected animal, has no influence on the phenomenon of enhancement. Animals solidly immune to both neuro- and testicle virus are as resistant to infections by the virus plus testicle extract as by the virus alone. Slightly autolyzed extracts are endowed with the same power as extracts recently obtained.

The virus does not seem to be modified in its virulence, for strains secured from the enhanced lesions are no more highly infective than those secured from the ordinary lesion. That, at least, the action of the extracts is directed toward the cell is shown by the occasional observation that some rabbits having pronounced lesions from pure neurovirus showed a typical vaccinal eruption in spots injected only with testicle extract. This suggested an experiment in which the shaved skin of a rabbit received in three different spots testicle extract,[5] and at the same time 1 cc. of neurovirus diluted with 20 cc. of Ringer's solution was injected into the ear vein. Typical lesions developed at the three injected spots and the animal died on the 4th day, showing that the blood virus localized only in the sensitized spots. After the injection of testicle extract the cells remained sensitized for a certain length of time. This was shown by an experiment in which 5 rabbits were injected intradermaly with 0.5 cc. of the extract while in the course of the following days 0.25 cc. of a 1:50 dilution of the virus was injected in the same spots. Another spot on the skin was injected with the virus alone in each case as a control. Enhanced lesions were

[5] The extract used in this experiment was kept 24 hours at room temperature and 24 more in the ice-box. Two similar experiments dealing with testicle virus gave negative results.

obtained up to the 3rd day, whereas later the lesions were not different from those obtained in the control spots, or the enhancement was doubtful. The virus used was of moderate strength, so that perhaps a longer sensitization would have been observed with a more active virus.

Preliminary Experiments on the Physical and Chemical Nature of the Enhancing Substance.—As the result of a number of preliminary experiments some of the properties of the enhancing substance have been determined. Dilutions of the supernatant fluids of testicle extracts, as high as 1:160 are almost as active in their enhancing power as the full strength extracts. Berkefeld filtrates of the extracts contain the enhancing substance in considerable amounts. Exposure to 100°C. for 3 minutes completely destroys the activity of the extracts. The precipitate resulting from the acidification of a Berkefeld filtrate of testicle extract contains practically all of the enhancing substance. This precipitate gives uniformly a strong Feulgen reaction, indicating a predominance of nucleoprotein elements.

DISCUSSION

The experiments reported here show that the neuro-, testicular and cow strains of vaccine virus are not only uninjured by contact *in vitro* with sensitive tissues from susceptible animals, but their infectivity is enhanced to an extraordinary degree by such contact. This finding is in strong contrast to the results obtained when a chicken tumor agent is subjected to similar treatment. The fact that one agent is either bound or inactivated by the constituents of the more susceptible tissue while another has its activity greatly enhanced suggests a fundamental difference in the mechanism involved in the action of the two and tends to separate them into different classes. It is known that certain enzymes form a union with the specific substance on which they act and this is supposed to be true in general for the enzyme-like agents. Whether or not the virus group is uniformly unaffected by contact *in vitro* with tissues of susceptible animals, or may have their activity enhanced by such tissues, remains to be determined. In the instance of the vaccine virus the evidence is conclusive.

The result of contact of vaccine virus with tissue extracts from immune animals and the chicken tumor agent with tissues from susceptible fowls is the same, namely, inactivation in both instances. The fact that vaccine virus is inactivated particularly by the sera from immune animals is well known and is generally considered to be the result of a real destruction of the virus. But the fact that the reaction between the virus and the immune tissue extract reaches its maximum almost immediately after the contact is effected and does not progress, and furthermore, because of the similarity between this effect and the inactivation of the chicken tumor agent by sensitive tissues, seemed to indicate that the action was not a destruction of the virus. The recent work of Andrews (6) has shown that the active virus may be recovered after contact with immune serum even when the serum is used in great excess. Furthermore Long and Olitsky (7) have shown that active vaccine virus may be recovered by cataphoresis from the testicle of immune animals many months after the disappearance of all active lesions. Hence it seems that the virus is not necessarily destroyed by the immune substance.

The most active of the tissues in the augmentation of the infectivity of the vaccine virus is the testicle. It is of interest to note that this organ is not only the most sensitive tissue to direct inoculation but is with the ovary the site where, in absence of previous irritation, the vaccine virus localizes most abundantly after intravenous inoculation.

The mechanism of the enhancement is not clear. The fact that the virus injected into an area of skin some hours or days after the tissue extract has been injected into the same area, results in an enhanced lesion, suggests that the effect of the extract is on the host cells, rendering them more susceptible to the virus, rather than on the virus direct. This interpretation is further strengthened by the observation that virus injected intravenously localizes most readily in areas of skin previously injected with testicle extract, and the lesions resulting are very extensive. It may well be that the extracts act primarily as a stimulus to cell division, thus increasing the number of young cells which are supposed to be more susceptible to the virus effect than older cells.

Aside from the organs or tissues whose extracts enhance the activity of vaccine virus there are others which frankly interfere with the infec-

tion. Among these latter the spleen pulp shows greatest inhibiting effects. In this connection evidence associating lymphoid reaction with the inhibition of tissue growth is recalled. But regardless of the explanation of the phenomenon described it seems that a basic difference has been established between the behavior of the filterable agent of the chicken tumor and a typical member of the so-called filter-passing viruses.

SUMMARY

Brain and testicle tissue from immune rabbits brought in contact with the Levaditi or Noguchi strains of vaccine virus will fix or inactivate the virus. Extracts of normal testicles from susceptible animals enhance to an extraordinary degree the infectivity of both the neuro- and dermal strains of vaccine virus. The Noguchi virus is not affected by testicle extracts when injected into the skin, but kidney extract has a definite enhancing power on the strain when injected into either skin or testicle.

The effect of tissue extracts seems to be on the cells of the host rather than on the virus. This is indicated by the fact that virus injected intravenously localizes most readily in an area of skin previously injected with testicle extract. Furthermore an enhanced lesion results if the virus is injected into an area as long as 3 days after the area has been injected with testicle extract.

The augmenting substance of the tissue extracts is little affected by high dilutions, passes through a Berkefeld V candle and is carried down with the proteins precipitated by weak acids.

Rabbits with enhanced lesions show general symptoms and about 25 per cent die with generalized vaccinia.

Kidney, and probably skin, brain and liver extracts possess enhancing properties, but to a much less degree than the testicle. On the other hand, spleen, blood and probably lymph nodes and bone marrow not only fail to produce enhancement, but actually restrain or even suppress entirely the vaccinal skin infection.

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EXPLANATION OF PLATES

PLATE 14

FIG. 1. (Rabbit 160, right side). Lesion produced by the intradermal injection 4 days before of 0.25 cc. of a 1:50 dilution of neurovirus plus 0.50 cc. of the precipitate obtained by the addition of acid to the Berkefeld and concentrated testicle extract.

FIG. 2. (Rabbit 160, left side). Control. Lesion produced by 0.25 cc. of the virus dilution plus 0.50 cc. of Ringer's. Note in the lower abdomen the oedema from the right side lesion.

FIG. 3. (Rabbit 163, right side).

1. Lesion produced by the injection 5 days before of 0.25 cc. of a 1:50 dilution of neurovirus plus 0.50 cc. of testicle extract.

2. Lesion produced by 0.25 cc. of the virus dilution plus 0.45 cc. of spleen extract.

FIG. 4. (Rabbit 163, left side). Control. Lesion produced by 0.25 cc. of a 1:50 dilution of virus plus 0.50 cc. of Ringer's.

PLATE 15

FIG. 5. (Rabbit 97, right side).

1. Lesion produced by the injection 8 days before of 0.25 cc. of a 1:50 virus dilution plus 0.50 cc. of testicle extract.

2. Lesion produced by the injection 8 days before of 0.25 cc. of a 1:100 virus dilution plus 0.50 cc. of testicle extract.

3. Lesion produced by 0.25 cc. of a 1:100 dilution of neurovirus plus 0.50 cc. of kidney extract.
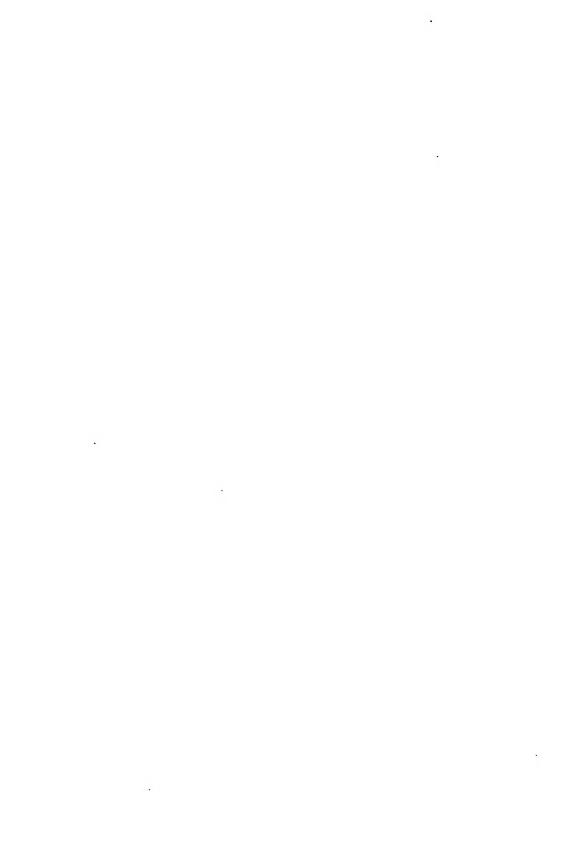
FIG. 6. (Rabbit 97, left side). Control.
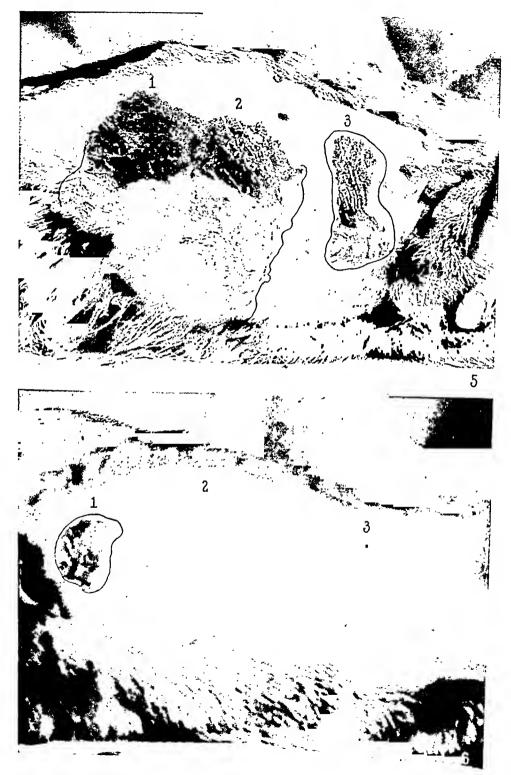
1. Lesion produced by the injection 8 days before of 0.25 cc. of a 1:50 neurovirus dilution plus 0.50 cc. of Ringer's.

2. Spot injected with 0.50 cc. of kidney extract alone.

3. Spot injected with 0.50 cc. of testicle extract alone. Note the secondary localization of the virus.

(The rabbit died 2 days after the photographs were taken.)

PLATE 14

A STUDY OF THE GENERALIZATION OF VACCINE VIRUS FROM ENHANCED SKIN LESIONS

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PLATES 16 TO 18

One of us has shown in a previous paper (1) that extracts from normal testicles are endowed with the power of enhancing to an extraordinary degree the lesions produced by vaccine virus. Extracts from kidney, liver, skin, brain and placenta share this property only to a certain extent. Extracts from suprarenal, retina, muscle and ovary do not modify the vaccinal infection, whereas blood, spleen and bone marrow extracts frankly interfere with and even suppress the activity of the virus.

Rabbits injected intradermally with mixtures of neurovirus and testicle extract become ill, develop a very high temperature, lose considerable weight, and approximately 25 per cent of the animals die from the disease. The present study is chiefly concerned with the histological characteristics of the wide-spread, typical vaccina, together with the non-specific lesions found at autopsy, and with the bearing of these findings on the much discussed question of the tissue affinities of the vaccine virus.

Calmette and Guerin (2) found that when vaccine virus was injected intravenously, it localized selectively in the shaved areas of the skin, whereas no localization was observed in mesodermic organs. This phenomenon was also observed by Camus (3), who found moreover that the virus localized in the buccal mucous membrane, genital organs and in cutaneous *naevi*. It was likewise noted by Levaditi and Nicolau, Rivers and Tillett, Noguchi and others (4, 5). Borrel and Burnet (6, 7) introduced the term *épithélioses* for a group of diseases produced by filter-passing viruses; viz., vaccinia, variola, fowl-pox, sheep-pox and molluscum contagiosum, all of which were said to attack epithelium almost exclusively. Lipschütz (8) promulgated the theory that the tissue localization of a virus is

conditioned by so-called specific affinities. A similar hypothesis was sustained by Menze (9). The doctrine of the *ectodermoses neurotropes*, however, has been defended mainly by Levaditi and his coworkers. Vaccine virus is regarded as a typical member of this group, showing a marked affinity for the internal segment of the ectoderm (neuraxis) and a more or less pronounced affinity for the external segment of the same ectoderm. The mesoderm and blood, therefore, would be considered wholly non-susceptible, and only certain entodermic organs would be involved.

In 12 experiments performed by Levaditi and Nicolau (10) vaccine virus was injected into rabbits, usually by the intravenous route. These rabbits were killed in from 6 to 10 days. The virus did not localize, or else was poorly localized, in mesodermic organs, whereas it did localize in the mammary glands, adrenals, tongue and buccal mucosa, and likewise in the skin and brain, but only after irritation. It localized abundantly in the lungs and moderately in the liver and the submaxillary salivary glands. Histological lesions, infiltrative or necrosing, were found in the mammary glands, and on two occasions in the lungs and liver. They were always absent in mesodermic organs. Of all the structures studied by Levaditi and Nicolau, the testes and ovaries were regarded as the most sensitive to vaccine virus because the virus could readily be recovered from them and because, at histological examination, necrotic lesions, interstitial in distribution, were consistently found.

The susceptibility of the testicle had already been shown by the work of Noguchi (11), Ohtawara (12), Rivers and Tillett (loc. cit.) and others, who found that the testis was the most suitable organ in which to demonstrate very small amounts of virus. Moreover, Parker (13), Merwin and Schemerling (14), and Eagles and McClean (15) reported data showing that testicular tissue is an excellent substrate for the *in vitro* cultivation of vaccine virus.

The concept of *ectodermoses neurotropes* was enunciated in 1921 (16). Since then there has been a reaction against it, since later experimental work has failed to bring supporting evidence. Thus, Blanc and Caminipetros (17), Chaumier (18), Walthart (19) and Ledingham and McClean (20) succeeded in implanting and passing vaccine virus in pure mesodermic tissues, such as muscle, kidney, lymphatic tissue and derma (corium) without any loss of activity. Moreover Ohtawara (loc. cit.) and Gildemeister and Heuer (21), after intratesticular inoculation, withdrew the virus from the blood as late as the 15th day after the injection. Recently Wilson and Smith (22) by the use of a special technique of fractionation, found the virus adherent to the white blood cells up to 8 days after injection. Vaccine virus has been successfully cultured *in vitro* in a kidney medium by H. B. and M. C. Maitland (23).

Watanabe (24), in a small series of animals injected intravenously, found that the virus localized especially in the dermis without involvement of the epithelial cells; it was likewise found in the liver and spleen.

A very extensive study of the subject has been made by Douglas, Smith and Price (25), who, after intravenous injections in a particularly sensitive strain of

rabbits, noted that the virus produced macroscopic specific lesions (pocks) in the following organs, arranged in order of their frequency of involvement: first, lung, skin and mucosa, spleen, and liver; secondly, genital organs and adrenals, both with the same percentage incidence of lesions. After intradermal and cerebral inoculation some generalization was detected, especially in the lungs, liver, skin and spleen. The tissues were tested by sub-inoculation for infectivity and it was found that during the first two weeks the virus was abundant in the lungs, spleen, bone marrow and genital organs; after this time, however, the skin, tongue, adrenals, ovaries and testicles seemed the most prone to harbor the virus. These authors' conclusion was that vaccine virus "possesses varying degrees of affinity for different tissues and general statements of tissue susceptibility to vaccinia virus should however be received with caution."

Ledingham (25) believes that the reticulo-endothelial system is in the main affected and responsible for the vaccinal infection, the epithelial lesions being secondary to the attack on the mesodermal derivatives. This conclusion was reached both by the histological study of the experimental lesion and by the fact that a blockade of the system in the skin with India ink frankly interfered with the infection. Several authors have already regarded the variolous pocks in the skin as being purely inflammatory. MacCallum and Moody (27) state that in alastrim the corium is involved before the epithelium.

The literature concerning the pathological anatomy in variola and allied diseases is most extensive. A complete account of the work previous to 1904 will be found in the memoirs of Councilman, Magrath and Brinkerhoff and their associates (28), but most of these papers are concerned with the parasitic nature of the virus and deal chiefly with skin and corneal lesions where Guarnieri bodies are easily found. Nevertheless the above-mentioned authors give a very complete description of the generalized lesions in variola, noting in the testes specific "anemic focal necrosis," and furthermore degeneration apparently not truly anemic in origin, but "due to the action of toxins," in the blood-forming cells of the bone marrow which constitute for them lesions almost pathognomic of variola but nevertheless devoid of "parasites." Chiari described these bone marrow lesions, applying the term "osteomyelitis variolosa." Councilman and his associates likewise noted the constant occurrence of more or less acute diffuse degenerative changes in liver, kidney, suprarenals and testes.

The most common lesion found in the lung and one which was very rarely absent was bronchitis, usually combined with more or less extensive bronchopneumonia even when this was not grossly demonstrable.

As early as 1886 Chiari (29) found in the testicles of 15 variolous children and in 85 per cent of adults with smallpox, typical foci with a central area of necrosis surrounded by an area of cellular infiltration. He regarded these lesions as being due to the direct action of the virus. Similar necrotic specific changes in the bone marrow and testes have been found by MacCallum (31) in alastrim. MacCallum mentions the intense cloudy swelling and focal necrosis in the liver, and the exten-

sive degenerative changes in the kidneys. In most cases there was an acute bronchitis, and in many there occurred a rather severe lobar pneumonia.

Material and Methods

The rabbits used were mostly of the common gray variety; some, however, were of the albino type. No essential difference between them could be detected. The age of the animals appeared of no significance.

The animals studied were grouped and treated as follows:—

A) Twenty-one rabbits were injected intracutaneously with neurovirus plus testicle extract. Twelve of these died as a result of the infection. The others were killed when in a more or less serious general condition. All of them were autopsied during the course of extensive specific lesions in the skin, the area involved often occupying the whole flank and abdomen.

B) Eight rabbits were injected intravenously with either 1 or 2 cc. of neurovirus diluted to 1:10 or 1:20, alone or mixed with rabbit testicle extract. Two of the animals died; the others were killed.[1]

C) Two rabbits were injected in the testis, two more in the brain and one directly in the liver.

The disease resulting from the injection of neurovirus plus testicle extract when inoculated together into the skin has already been described (1).

Gross Lesions

At the postmortem examination of the animals dying from vaccinia the following changes were observed:

A markedly edematous zone of hemorrhagic character widely surrounded the borders of the vaccinial lesion in the skin. The lymph nodes of the drainage area were very much congested and enlarged. There was marked congestion of the splanchnic organs—kidneys especially; the liver was enlarged. The peritoneal cavity frequently contained a few cubic centimeters of viscid hemorrhagic fluid. The muscles of the abdominal wall occasionally showed discrete punctate hemorrhagic areas. There was uniformly congestion of the testes with some fluid in the tunica vaginalis. The most obvious gross changes were found in the lungs where

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[1] The virus was prepared as described in other papers. This particular sample was of a uniform activity, giving positive takes in the skin at a dilution as high as 1:2.000.

the lesions consisted of foci of congestion and patches of lobular pneumonia. We are fully aware of the doubtful significance of such lesions in the rabbit lung. The pleurae and pericardium were occasionally involved, the serous cavities being distended by fluid.

In the animals killed the intensity of the lesions bore a comparable relationship to the gravity of the clinical condition.

Pocks in internal organs, as described by Douglas, Smith and Price, were never encountered. Only in the suprarenals and ovaries the existance of clean-cut and more or less hemorrhagic foci with superficial elevation of the gland surface enabled one to make the diagnosis of vaccinal infection macroscopically. The testicles likewise showed these foci, but less clearly. Skin generalization was observed but very rarely. In more than 200 rabbits with simple or enhanced cutaneous or testicular lesions, typical pocks were encountered with some regularity, although in small numbers, in the tongue and lips alone.

It must be kept in mind that we used the same strain of virus that Douglas, Smith and Price used, so that evidently idiosyncrasy in the rabbits is of paramount importance as regards the generalization and character of lesions produced by the neurovirus.

Microscopical Findings

Material was fixed in Zenker's fluid and thin sections—some 4 micra in thickness—were stained in Mallory's eosin-methylene blue, phloxine-methylene blue, or by Wolbach's modification of the Giemsa stain. Lesions regarded as specifically vaccinal were found by histological examination in lung, liver, spleen, lymph nodes, bone marrow, adrenal, ovary, and testis; one doubtfully specific lesion was encountered in the kidney. Changes considered as accessory and not definitely specific were found in spleen, lymph nodes and kidneys. In the specific visceral lesions nothing of the nature of Guarnieri bodies were found after long search, save perhaps in the ovary, where, in two animals, structures were present in Graafian follicle cells adjacent to an active vaccinal necrosis, seemingly cytoplasmic inclusions answering the histological criteria for the Guarnieri body. Both the characters and the distribution of the vaccinal lesions leave little doubt as to their specific nature. The characters are obvious from the subsequent descriptions. The distribution is not in agreement with that of any known type of accessory streptococcic invader since the organs affected mostly are those generally shunned by the blood-born streptococcus. Furthermore, a check on the vaccine content of the organ by subinoculation and a comparison between the organ content of virus and the blood content in the same animal would apparently eliminate any doubts.

Liver: Liver lesions occur in diverse forms. In the earliest foci the walls of the liver sinusoids are damaged and an exudate of precipitated albumen and fibrin occupies the space between endothelium and liver parenchyma. One might be

led to infer from these early capillary lesions, which are promptly followed by the appearance of fibrin plugs within the vessels, that the subsequently appearing, abscess-like foci are of thrombotic origin. However, before capillary thromboses occur even in the earliest lesions, one finds one or more punctate liver cell necroses, and it is the punctate character of these necroses which forces one to eliminate thrombosis as a causative factor in their genesis. The lesion is primarily paren-chymatous. These necrotic liver cells undergo cytoplasmic hyalinization; they stain deeply pink with eosin-methylene blue or Giemsa stains; their nuclei become pyknotic or gradually fade out, the chromatin being reduced to a fine dust-like deposit. Neither nuclear nor cytoplasmic swelling ("ballonartige") has been seen.

As the lesion enlarges the capillaries become plugged over a wider area and the thrombosis may even extend into larger vessels. It is particularly in this case that one may be led to regard the lesion as thrombotic; however analysis forces us to abandon this suggestion since in several instances it has been possible to trace these wide-spread areas of hyalin necrosis and to follow their formation as fusions of originally punctate single cell necroses such as those shown in Fig. 1. They occur in rabbits grossly free from evidences of coccidiosis or verminous cysts. One noteworthy feature is the very low grade reactive process; a few polymorphonuclear leukocytes may appear in the thrombosed sinusoids and may invade the necrotic liver cells, but the appearance is that of a paralyzed, insignifi-cant type of reaction; the leukocytes present exhibit marked fragmentation of their nuclei which rapidly degenerate into nuclear dust. Fibroblastic proliferation is absent in the acute stage. Guarnieri bodies have never been seen.

Suprarenal: The lesions of the suprarenal are quite similar to those of the liver, save that the broad zones of hyaline necrosis were but once encountered. The early foci are either of the nature of sharp punctate single cell necroses or isolated hemorrhagic foci of varying size. (Fig. 2). The parenchymatous cells undergo rapid hyaline degeneration; a fibrin exudate is thrown out about the capillaries; fragmented polymorphonuclear leukocytes invade the necrotic cells. The reaction may become fairly intense, whereupon the appearance is that of an abscess with intense necrosis. (Fig. 3). In the early stages there is evidence of stimulation of the suprarenal parenchyma and several mitotic figures per oil immersion field may be encountered in pink-staining, damaged cells. (Fig. 5). Ballooning degeneration has not been seen and no Guarnieri bodies were to be found. These lesions are not due to secondary bacterial invaders; they appear in animals injected intravenously or intratesticularly or directly into the liver—in animals without skin lesions to serve as a source for secondary microbic invasion.

Ovary: The ovarian lesions are widely distributed throughout all anatomic divisions of the organ. They are to be found in the stroma, they involve the interstitial cells, theca interna and externa, and corona radiata. They are of the usual hemorrhagic, necrosing, degenerative type (Fig. 4) with much fibrin deposit, and may call forth a considerable polymorphonuclear and monocytic reaction. In the ovary alone of all the viscera have structures been encountered which

answer all the histological requirements of the Guarnieri bodies. These cyto-plasmic inclusions are pictured in Figure 6. One must emphasize, however, the difficulty of really interpreting these structures; necrosis is so acute and so exten-sive, cell fragmentation so wide-spread, bits of broken-up acidophilic cell detritus are so readily washed from place to place during the mere technical handling of material, that appearances may become very deceptive. We feel therefore some doubt as to the nature of the structures which answer the morphological require-ments of Guanieri bodies, especially in view of the fact that in no other viscus have they been certainly encountered.

Testis: Testicular lesions involve tubules, interstitial tissues, and in one instance the tunic was affected, with the production of an acute vaccinal periorchitis. These specific lesions in no way differ from those of the ovary or suprarenal. Specific cell inclusions could not be identified. In addition, in many animals in which no specific testicular pustules were found, spermatogenesis was nevertheless in abeyance, a certain amount of isolated punctate sex cell necrosis was observed and although we are unable to state that these lesions are specific, they never-theless are undoubtedly related to the vaccinia or to the generalized intoxication accompanying the disease. In the summary showing percentage incidence of lesions in various organs, these minor testicular findings have been regarded as non-specific and are therefore not included. Slight sex cell necrosis has been encountered in controls.

Lungs: In the lung the vaccinal lesions may readily be confused with those of rabbit pneumonia. They differ from the common pneumonic lesion in several respects: they are more sharply focal; they are not primarily associated with bronchi but rather with vessels. They occur as a pronounced inflammatory, necrosing process in the vessel wall, with edema, thick perivascular deposition, invasion of the vessel wall by polymorphonuclear leukocytes, greatly fragmented and largely necrotic. From the vessel the lesion spreads to the neighboring alveoli but is apparently slow in giving rise to exudates within the bronchi. The nature of the process is shown in Figure 9. Occasional localized pleural and subpleural lesions of the same type exist. The typical lung lesions have been but rarely encountered. In one animal an almost tubercle-like, sharply focal necros-ing lesion was found, a necrosis with practically no reaction on the part of inflam-matory cells and with no involvement of the bronchi. Trachea and esophagus have been uniformly negative.

Spleen: The splenic lesions do not partake of the pustular character of the typical vaccinal foci encountered in the lungs, liver, adrenal, ovary, testis, nor the dermal pocks. The most striking thing about the spleens is the very marked lymphocytic exhaustion. The Malphigian bodies are scanty and small; the pulp lymphocytes are reduced in number; occasionally germinal centers are seen without any surrounding zone of small lymphocytes. There may be necrosis of splenic capillaries with thick perivascular fibrin deposits (Fig. 7). Isolated cell necrosis,—of lymphocytes, polymorphonuclear leukocytes, and large phagocytic cells may

be found. Hemorrhage is common. Where necrosis has been present the splenic lesions have been classified as vaccinal; in spleens where the only feature has been the pronounced lymphocyte exhaustion the lesion has been regarded as accessory only. Numerous sub-inoculations with spleen might be required before these doubtful points could be decided. Lymph nodes contain lesions similar to those of the spleen and have been similarly regarded. Fig. 8 indicates the extensive necroses which may be encountered in the lymph nodes.

Bone Marrow: We have studied but one bone marrow. This marrow showed an extensive necrosing vaccinal osteomyelitis with destruction of all marrow elements. Very little reaction was present in comparison with the intensity of the necrosis. The reacting polymorphonuclear leukocytes were greatly fragmented.

Kidney: No specific lesions have been encountered in the kidneys examined. Nevertheless acute degenerative changes in the convoluted tubules and collecting tubules are very common. These changes consist in swelling, albuminous degeneration, the presence of tubule casts, and in one instance a rather puzzling lesion, provisionally interpreted as an infarct, but nevertheless a peculiar one, for instead of a sharp zone of necrosis with surrounding reaction, the necrosis was poorly outlined and isolated necrotic tubules extended in fan-like processes far outward from the zone of major necrosis into the surrounding region of normal tubules. Leukocytic reaction was conspicuous by its absence. It is therefore possible that this lesion was specific.

Percentage Incidence of Visceral Lesions

In rabbits bearing enhanced skin lesions the percentage incidence of visceral lesions is summarized in Table I.

TABLE I

Specific Necrotic Lesions in Rabbits with enhanced skin lesions

Organ	Number Examined	Number with Specific Lesions	Percentage
Ovary	4	3	75.0
Suprarenal	11	8	72.7
Lymph Nodes (Vicinity of Lesion)	11	6	54.5
Testicle	13	7	53.8
Spleen	15	6	40.0
Lung	21	4	19.4
Bone Marrow	1	1	100.0
Kidney	15	1*	6.6
Brain	8	0	0.0

* Doubtful.

Accessory lesions in rabbits with enhanced skin lesions have been found in 10 cases in the spleen (66 percent), (lymphatic depletion), in 13 cases in the lung (60 percent), and in 9 cases in the kidney (60 percent). It must be added that both specific and accessory lesions were present in practically 100 percent of rabbits dying of vaccinal infection. The lowering of this percentage is due to the introduction of figures from rabbits purposely killed, some of them while in a good general condition.

The results in animals injected intravenously, intratesticularly or into the liver are summarized in Table II.

TABLE II

Specific Necrotic Lesions in Rabbits Injected into Liver, Testis, and Brain

Organ	Number Examined	Number with Specific Lesions	Percentage
Ovary	1	1	100.0
Liver	7	3	42.9
Testicle*	10	4	40.0
Suprarenal	8	2	25.0
Spleen	8	2†	25.0
Lung	9	0	0.0
Kidney	10	0	0.0
Brain	1	0	0.0

* In the case of intratesticular inoculation, the lesions of the opposite testicle only have been taken into consideration.

† One doubtful.

Accessory lesions in these rabbits have been found in 6 cases in the lung (66 percent), 6 cases in the kidney (60 percent), and 5 cases in the spleen (63 percent), (lymphocytic depletion). In two rabbits injected into the brain and dying 3 days later, very wide-spread accessory lesions were found in the kidney and also in the intestine.[2]

In a total of nine rabbits, of which 5 were injected intravenously and killed after 4, 6, 7 and 9 days, in two animals dying with enhanced skin lesions and in two others dying 6 days after intratesticular injection, the organs were tested for the presence of the virus by applying

[2] The lymph nodes were never examined as there was no gross indication of any lesion whatsoever.

the various organs to the scarified skin of normal rabbits. That virus could be recovered from internal organs is shown in Table III.

TABLE III

Recovery of the Virus

Organ	Number Examined	Number from which Virus Recovered	Percentage
Ovary..................................	1	1	100.0
Kidney.................................	9	5	55.6
Testicle................................	8	4	50.0
Suprarenal.............................	9	4	44.5
Liver..................................	9	4	44.5
Lung..................................	9	3	33.3
Spleen.................................	7	2	28.9
Blood.................................	9	1*	12.2

* Very weak eruption.

The histological examination of the same fragment of the organ which was tested for its virus content was in general agreement with the result of the inoculation. Nevertheless, there were some discrepancies, for virus was recovered from lung and spleen in three cases, where no lesions, specific or accessory, were found, and in three other instances lesions in liver, kidney and suprarenal were found, whereas no virus was recovered. Either the inhibitory effect of the organ or the possible antibody content may explain the apparent discrepancy. In three of the rabbits of injected intravenously, the neurovirus was inoculated after mixing with 2.0 cc. of rabbit testicle extract prepared as described in a previous paper (1). A very careful study of the organs of these rabbits from both the histological and the virus content point of view did not show more infection than in animals injected with the virus alone. Testicle extract therefore does not appear to enhance the action of intravenously injected virus.

DISCUSSION

It would be quite apparent that any statements as to organ susceptibility to vaccine virus must take several factors into consideration. If we test for the virus content of an organ by applying the organ to

the scarified skin of a normal rabbit we obtain a certain result. However we may be in error since the enhancing or inhibiting action of various normal organ extracts is so marked that these activities may be of more importance in influencing the take in the normal animal than is the actual virus content of the tested organ. Again in all probability when testing an organ for virus content we are inoculating a mixture of virus plus antibody; the antibody content may not be the same in all organs; the rate of antibody formation is perhaps quite different. Were one to separate by cataphoresis methods the virus from the antibody in the various organs, then quite a different organ virus content might be ascertained. However that may be, the simple application of the suspected organ to the scarified skin of the normal rabbit may give one index of so-called organ susceptibility. Were it possible to show that the enhancing or inhibiting effects of different organs are paralleled by the multiplication or suppression of virus within the living cells of these organs then one might adopt quite a different standard of organ susceptibility. Finally, the presence of specific histological lesions in different organs would seem to offer still a third criterion for estimating specific organ affinities.

Our charts as well as the results of Douglas, Smith, and Price demonstrate that nowhere does vaccine virus exhibit any clear-cut, selective affinity for organ groups of similar embryological origin. Every organ reacts to vaccine virus in its own peculiar manner. An organ may appear insusceptible so far as the actual presence of specific histological lesions is concerned, and yet, extracts of it enhance the action of vaccine virus when mixed therewith *in vitro* and injected into the skin of a normal rabbit; another organ may manifest specific vaccinal lesions, yet, *in vitro*, a similar normal organ may inhibit the action of the virus; an organ may contain virus but exhibit no histological lesion or may present a specific histological lesion but prove negative on sub-inoculation. Of all organs studied the testis alone yields a high percentage of specific lesions, gives a high percentage of takes on sub-inoculation, and has a marked enhancing action when its extract is mixed with vaccine virus and inoculated into the skin of normal rabbits. It is by far the most active of the organs we have tested.

SUMMARY

Rabbits bearing very intensive skin lesions resulting from the intracutaneous injection of neurovirus plus testicular extract show typical histological alterations in the gonads, suprarenals, liver, spleen, lung, lymph nodes, and bone marrow.

Similar but less wide-spread alterations are found after intravenous injection of neurovirus.

Although testicle extract injected intracutaneously with neurovirus has a marked enhancing action upon the activity of the latter, the same mixture injected intravenously yields no sign of any such enhancement.

The significance of these observations as regards the question of the ectodermotropism of vaccine virus is discussed, and the doctrine of specific organ affinities is considered.

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EXPLANATION OF PLATES

PLATE 16
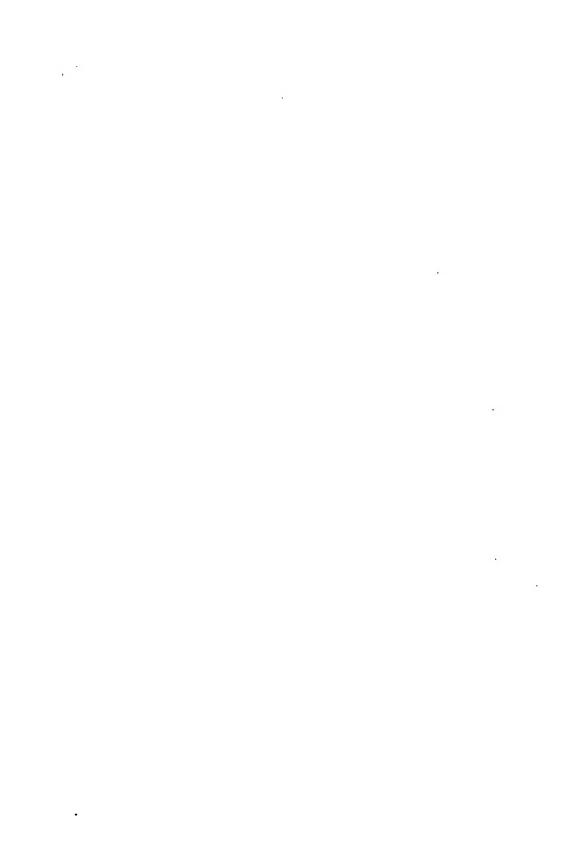
FIG. 1. Liver. Zenker; phloxine-methylene blue. Focal hyaline liver cell degeneration.   × 200.

FIG. 2. Suprarenal. Similar fixation, staining and magnification. Specific necrosing, hemorrhagic lesion.

FIG. 3. Suprarenal. Similar fixation and staining.   × 53.   Multiple specific, abscess-like, vaccinal lesions.
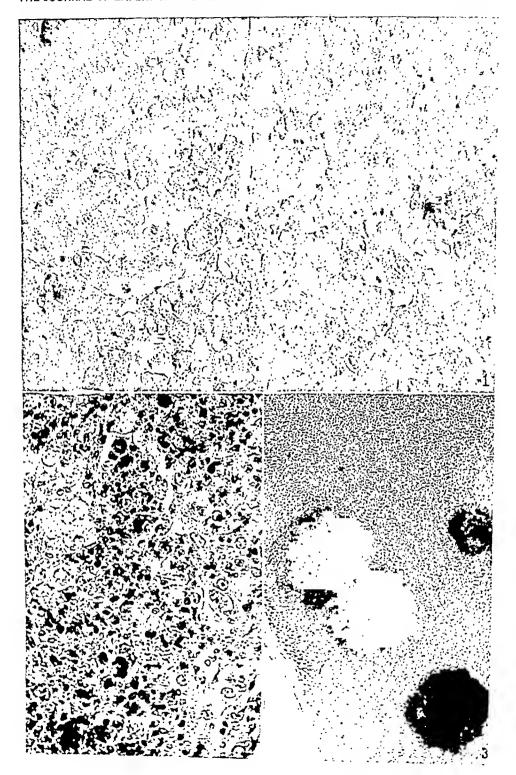
PLATE 17

FIG. 4. Ovary. Zenker; Giemsa. Hemorrhagic, necrosing lesion involving Graafian follicle and theca.   × 110.

FIG. 5. Suprarenal. Zenker; Giemsa. Early necrosing lesion showing four mitotic figures as evidence of stimulation.   × 220.

FIG. 6. Ovary. Zenker; Giemsa. Probable Guarnieri bodies.   × 440.

PLATE 18
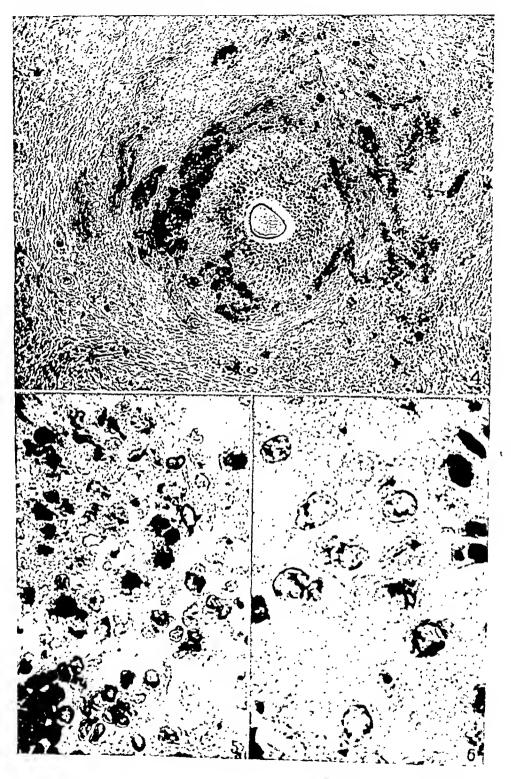
FIG. 7. Spleen. Zenker; phloxine-methylene blue. Diffuse fibrin deposit about vessels; marked diminution in lymphocytes.   × 110.

FIG. 8. Lymph node. Zenker; eosin-methylene blue. Diffuse necrosing lesion. Node taken from drainage area of an enhanced skin lesion.   × 53.

FIG. 9. Lung. Zenker; phloxine-methylene blue. Vascular and perivascular lesion interpreted as specific.   × 200.
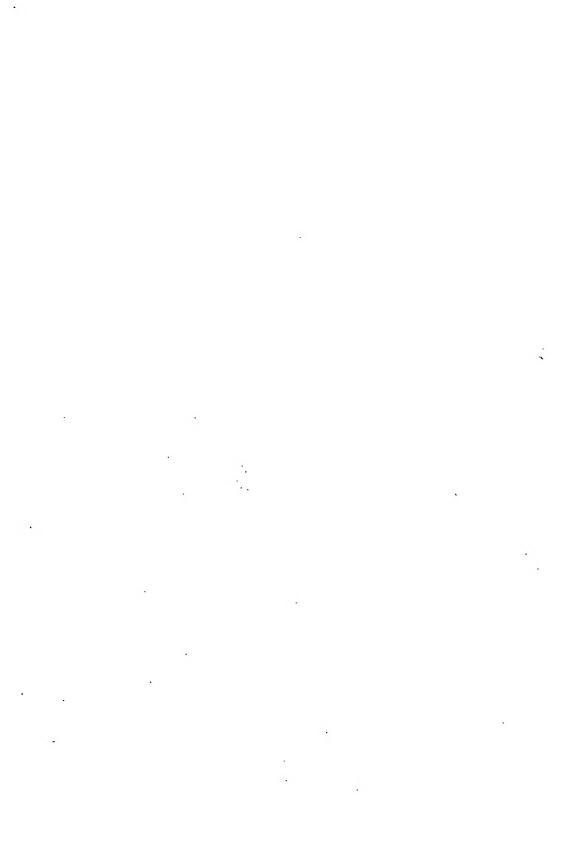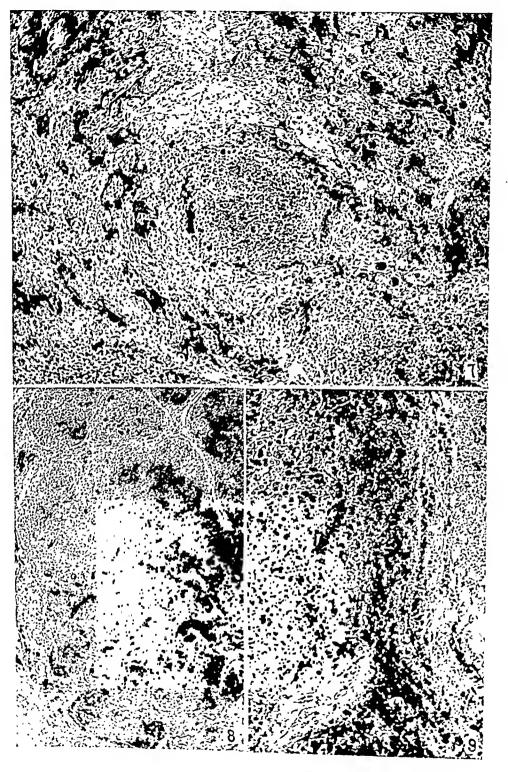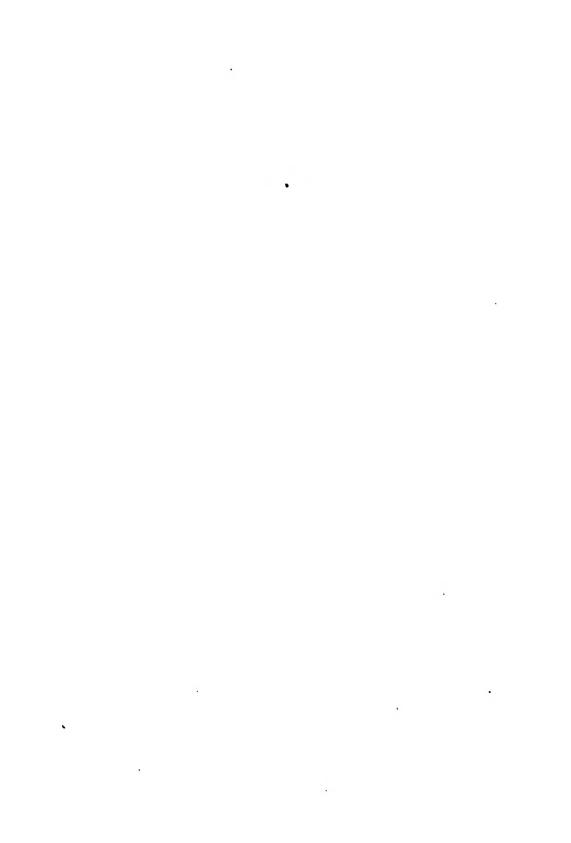
·ETIOLOGY OF OROYA FEVER

XV. Effect of Immune Serum on the Course of Bartonella bacilliformis Infection in Macacus rhesus

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PLATES 19 AND 20

Very early in the course of the work on Carrion's disease, experiments were begun (Noguchi) to test the effect of an immune serum, prepared in rabbits, or obtained from recovered monkeys, on the course of experimental infection with *Bartonella bacilliformis* in *Macacus rhesus*. While these early results were not altogether promising, owing probably to the use of small doses of immune serum, there was some indication that the introduction of the serum simultaneously with the infective material inhibited the development of the infection, at least, temporarily (Table 1), the controls showing skin lesions and positive blood culture earlier than the treated animals.

Recently, studies were undertaken to determine the effects of larger doses (20 cc.) of convalescent serum given 24 hours before intradermal and intravenous inoculation of a highly virulent strain of *Bartonella bacilliformis* isolated from phlebotomi.[1] This procedure was found to have a marked inhibitory effect (Table 2). While the control animal developed severe skin lesions within 2 weeks, and its blood yielded cultures of *Bartonella bacilliformis* in a dilution of 1:10,000 after periods of 10 and 17 days, the treated animals remained free from lesions for 25 days, and cultures of the blood were sterile. However, in two of the three treated animals, typical nodules eventually developed (after 25 days) at one or more sites of intradermal inoculation, and after 26 days the blood of one animal yielded cultures in a titre of 1:100. The third treated animal escaped infection.

[1] Noguchi, H., Shannon, R. C., Tilden, E. B., and Tyler, J. R., *J. Exper. Med.*, 1929, 49, 993.

TABLE 1

M. rhesus No.	Date of inoculation 1926	Material inoculated	Mode of inoculation	Result		Treatment Date Material	Result	
				Local lesions	Blood culture		Local lesions	Blood culture
59	Oct. 14	0.5 cc. susp. nod. Rh. 54 + 1.5 cc. immune rabbit serum. 2 cc. serum intrav.	Intradermal. Scarification.	No reaction until 20 days	− 5 days + 1:100,000 20 days	Nov. 26 3 cc. immune rabbit serum	Regression began Dec. 2	− Nov. 26 − Dec. 4
60	" "	Same except only 1 cc. serum intrav.	Same	Sl. transient reaction at one site after 20 days	− 5 days + 1:1,000 20 days			
61	" "	0.5 cc. susp. nod. Rh. 54 + 1.5 cc. convalescent serum Rh. 18 2 cc. serum intrav.	Same	No reaction	− 5 days Died of secondary infection Oct. 31.			
3-T	" "	Same as 61, but 1.5 cc. serum intrav.	Same	All inoculations positive in 12 days, mature nodules 20 days	+ 1:100,000 5 days + 1:100,000 20 days			

No.	Date	Inoculum	Site	Reaction	Serology	Remarks	
57 Control	Oct. 14	Mixture 0.5 cc. nodule susp. Rh. 54 and 1.5 cc. *normal rabbit serum*	Same	Definite 9 days	+ 1:100 5 days — 43 days	Nov. 26 1.5 cc. immune rabbit serum and 1 cc. culture subc.	No change Nodules still present Dec. 13 / — Dec. 4
2-T Control	" "	Same	Same	Definite 5 days	+ 1:100,000 5 days	See Etiology of Oroya Fever. XIII.	
1-T Control	" "	Nodule susp. Rh. 54	Same	Definite 8 days Well devel. 22 days	+ 1:1,000 5. days — 43 days	See Etiology of Oroya Fever. XIII.	
55 Control	" "	Same	Same	Definite 8 days. Well developed 13 days	— 5 days — 43 days		
83	1927 Feb. 8	Susp. nod. tissue *M. rhesus* 78 Cultures P-5 strain 5 cc. immune serum intrav.	Intradermal, eyebrows and abdomen	No reaction	— 9 days — 23 "	Reinoculated Mar. 16, 1927, with suspension nodular tissue of *M. rhesus* 3A. No reaction.	
84A	" "	Same	Same	" "	— 9 days — 23 "	Killed because of tuberculosis Mar. 12, 1927.	
82	" "	Control, no serum	Same	" "	— 9 days — 23 days	" "	
76	" "	Control, no serum	Same	Definite nodules 21 days. Reached 0.5 cm. in diameter	— 9 days + 1:100 23 days	" "	

TABLE 2

Effect of One Intravenous Injection of Convalescent Serum 24 hrs. Prior to Inoculation

M. rhesus No	Pooled convalescent serum cc.	Date of inoculation 1928	Material inoculated	Mode of inoculation	Result		
					Local lesions	Blood culture	
I-20	20	Nov. 10	Suspension nodular tissue from M. rhesus I-9 (Phleb. Str. 1)	Intradermal, 4 sites on abdomen. Scarification, 1 site Intrav. 1 cc.	One intradermal inoculation definitely positive after 25 days. Scarified area showed sl. reaction. Other 3 sites neg.	− 10 days − 17 " − 26 "	
I-21	20	" "	Same	Same	All inoculations negative	− 10 days − 17 " + 1:100 26 days − 33 days	
I-30	20	" "	Same	Same	2 intradermal positive after 25 days, 8 mm. in diameter.	− 10 days − 17 " − 26 " − 33 "	
I-31	Control, no serum	" "	Same	Same	All intradermal inoculations positive in 14 days, reaching full development in 25 days. Severe reaction on scarified area. Died Dec. 10 (30 days)	+ 1:10,000 10 days + 1:10,000 17 "	

Apparently the microorganisms had, in the two instances, remained dormant at the sites where they were intradermally introduced until the lapse of the period of passive immunity. This conclusion has been confirmed, by another experiment, as likewise the view that blood invasion was inhibited.

Four animals received an injection 24 hours before inoculation of 20 cc. of convalescent serum, another injection of the same amount 11 days after inoculation, and two subsequent injections of 4 cc. and 5 cc., respectively, 15 days and 26 days after inoculation. The control animals developed definite nodules in 11 days, which progressed rapidly and had reached very large size 12 days later. At this time the treated animals were still free from any lesions, and the blood was sterile. These conditions were maintained for a period of 38 days after inoculation (13 days after the last serum injection). At the end of this period, although blood cultures were still negative, definite nodules appeared at the sites of intradermal inoculation in 3 of the 4 monkeys; in the fourth animal nodules appeared 5 days later. The development of the lesions thereafter followed the usual course of experimental verruga, but *Bartonella bacilliformis* was recovered from the blood of the treated animals only in one animal (S-4) and then only once, 61 days after inoculation.

The experiment is recorded in detail in Table 3. It will be noted that the material used for inoculation was varied. Each monkey was injected intradermally at 3 or 4 sites on one side of the abdomen with a suspension of nodular tissue from a monkey infected with Phlebotomus Strain 1 of *Bartonella bacilliformis*, and each received similar inoculations on the other side with cultures of Phlebotomus Strain 3 or Strain 4. While the control animal for the nodule suspension responded in the usual way, only one of the 3 treated animals reacted to this material, though all showed the delayed reaction to the culture inoculations. But for the precaution taken to use varied material, the cultures in this instance proving to be of the maximum virulence, the results might have been interpreted as indicating that 3 of the 4 serum-treated animals were completely protected against infection. The one monkey which showed a delayed reaction (58 days) to the inoculation of the moderately virulent nodule suspension was evidently the most susceptible animal of the four, since it was the only one which was not completely protected against invasion of the blood by *Bartonella bacilliformis*

The results recorded in Tables 2 and 3 indicate that while the

TABLE 3

Effect of Treatment with Convalescent Serum (First Dose Given 24 Hours Before Inoculation)

M. rhesus No.	Pooled convalescent serum cc. total	No. injections	Material and mode of inoculation	Local lesions	Blood culture
S-1	49	4	*Nodule susp. rhesus I-30* 3 sites intradermally	—	— at 11, 19, 26, 46, 61 days after inoculation
			1 by scarification	—	
			Cultures Phleb. Str. 1 3 sites intradermally	Very slight induration 39 days Mature in 66 " Recovery complete in 83 "	
			1 by scarification	—	
S-2	Same	Same	*Nodule susp. rhesus I-30* 3 sites intradermally	—	— at 11, 19, 26, 46, 61 days after inoculation
			1 by scarification	—	
			Cultures Phleb. Str. 3 3 sites intradermally	Definite nodules 39 days Mature in 51 " Recovery complete in 83 days	
			1 by scarification	+	
S-3	Same	Same	*Nodule susp. rhesus I-30* 3 sites intradermally	—	— at 11, 19, 26, 46, 61 days after inoculation
			1 by scarification	—	
			Cultures Phleb. Str. 4 3 sites intradermally	Very slight induration 39 days Mature in 66 " Recovery complete in 83 "	
			1 by scarification	—	

			Inoculation	Results	Reaction
S-4	Same	Same	*Nodule susp. rhesus I-30* 3 sites intradermally; 1 by scarification	Nodule at 1 site 58 days / Mature in 98 " / 2.5 cm., pedunculated / Still 8 mm. at 111 days / —	— at 11, 19, 26, and 46 days, / + at 61 days after inoculation
			Cultures Phleb. Str. 4 3 sites intradermally; 1 by scarification	Nodules at 2 of 3 sites in 46 days / Mature in 83 " / Recovery complete in 102 days / —	
S-5 Control	None		*Nodule susp. rhesus I-30* 4 sites intradermally; 1 by scarification	Definite nodules in 11 days / Mature in 23 " / Recovery complete in 58 days / ++	+ at 11, 19, and 26 days, / — at 46 days after inoculation
S-6 Control	None		*Cultures Phleb. Str. 3* 4 sites intradermally; 1 by scarification	Definite nodules in 11 days / Mature in 23 " / Recovery complete in 65 days / +++	+ at 11, 19, and 26 days, / — at 46 days after inoculation
S-7 Control	None		*Cultures Phleb. Str. 4* 4 sites intradermally; 1 by scarification	Definite nodules in 11 days / Mature in 23 " / Recovery complete in 58 days / ++	— at 11 days / + at 19 and 26 days / — at 46 days after inoculation

TABLE 4

Effect of Treatment with Convalescent Serum (First Dose Given 5 Days after Inoculation)

M. rhesus No.	Material and Mode of inoculation	Date 1929	Local lesions	Blood culture	Amounts of convalescent serum administered
S-8	Culture, Phlebotomus Strain 4.	Jan. 23	First definite nodule (4 mm.) 5 days after inoc. Nodules 10 mm., red, prominent, scarification +, 13 " " Nodules average 15 mm., scarification + 19 " " Nodules mature (10–20 mm.) 36 " " Regression complete 67 " "	+ 5 days — 13 " — 30 " — 43 "	20 cc., 5 days after inoc. 20 cc., 13 " " 10 cc., 16 " "
S-9	Same		First definite nodule (4 mm.) 5 " " Nodules 8–9 mm. 13 " " Same, scarification + 19 " " Nodules mature (13–15 mm.) 36 " " Regression complete 67 " "	Same	Same

convalescent serum was not potent enough to protect completely against the inoculation of highly virulent cultures, it had a marked inhibitory action, as shown by the long delay in the development of the lesions, and that only occasionally did it fail to prevent invasion of the blood by the Bartonella.

The effect of large doses of convalescent *rhesus* serum given after local lesions had started to develop following the inoculation of cultures, was tested as follows:

In two *rhesus* monkeys, S-8 and S-9, treatment with convalescent serum was started 5 days after intradermal inoculation with a virulent strain of *Bartonella bacilliformis*, that is, when definite nodules had appeared, and when blood culture in both animals was positive in a dilution of 1:10. The serum was given intravenously in a dose of 20 cc. Notwithstanding the treatment, the nodules progressed rapidly, but 8 days later blood cultures were negative. At this time a second intravenous injection of 20 cc. of convalescent serum was given to each animal. Blood cultures continued negative throughout the remaining course of disease, but the local lesions progressed, and each animal was given another intravenous injection of 10 cc. of the immune serum on the 17th day. There was no perceptible effect of the serum treatment on the growth of the nodules, which matured about the 36th day after inoculation. Recovery was complete on the 67th day. At no test after serum was given were positive blood cultures obtained.

This experiment, recorded in detail in Table 4, shows that convalescent serum, given after the development of the nodules, sterilized the blood stream but had no effect on the nodule formation.

SUMMARY

Experiments are reported on the effect upon the course of experimental verruga peruana in *Macacus rhesus* of the injection of (1) small quantities of rabbit immune serum simultaneously with living cultures, (2) one large dose of convalescent monkey serum 24 hours prior to inoculation, (3) a similar preliminary dose followed by three subsequent injections of the serum, (4) three large doses of convalescent serum, following the inoculation. The convalescent serum was found (1) to prevent the multiplication of *Bartonella bacilliformis* in the blood in most instances, and (2) to delay the development of the skin lesions for considerable periods, when given before inoculation. When the serum treatment was not begun

until after the appearance of the skin lesions, it had no effect on the progress of the nodules, although the blood became free from *Bartonella bacilliformis*.

Since the severe effects of verruga peruana (Carrion's disease) are believed to be due to the multiplication of *Bartonella bacilliformis* within the blood, the injection of convalescent serum in cases of Carrion's disease in man would appear to offer promise.

EXPLANATION OF PLATES

PLATE 19

Figs. 1 and 2.   Two of the control animals, S-7 and S-5, 24 days after inoculation.

Figs. 3 and 4.   Treated animals, S-1 and S-2, 24 days after inoculation.   Compare with Figs. 1 and 2.
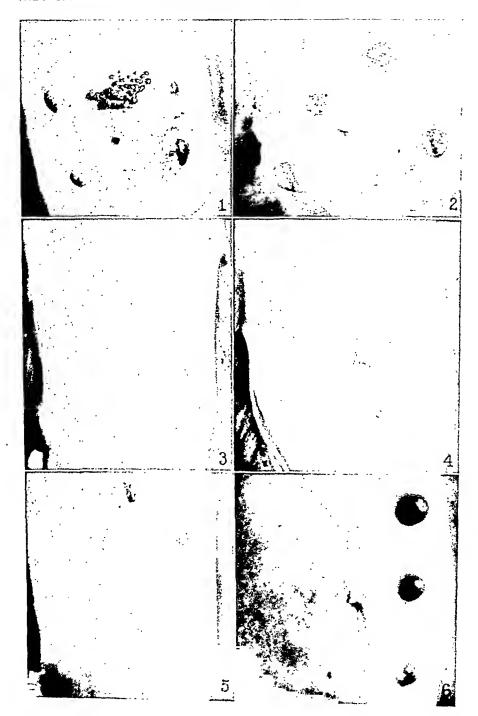
Figs. 5 and 6.   Treated animals, S-1 and S-2, 54 days after inoculation, when the lesions were comparable with those of the controls at 24 days (Figs. 1 and 2).

PLATE 20

Figs. 7 and 8.   Treated animal, S-5, 24 and 54 days after inoculation, respectively.
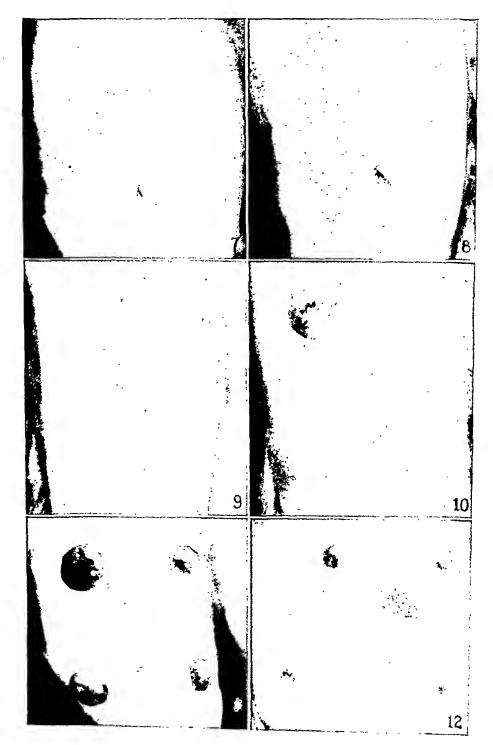
Figs. 9 and 10.   Treated animal S-4, 66 days and 100 days after inoculation, respectively.   At 66 days the lesions had just become definite.

Figs. 11 and 12.   Rhesus S-8 and S-9 (treatment begun after development of the lesions), 22 days after treatment was begun.

A PARALYTIC DISEASE OF GUINEA PIGS DUE TO THE TUBERCLE BACILLUS

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In a recent investigation in which a large number of tuberculous guinea pigs were under observation, a relatively high percentage of the animals became paralyzed early in the disease. These animals had each received subcutaneously 0.1 mg. of a culture of tubercle bacilli designated P. J. Out of 285 animals in the group between 30 and 40 manifested the paralysis. It had been noted previously among our tuberculous guinea pigs, but the cases had been so infrequent and scattered as not to attract serious consideration. In this particular experiment the disease complex made its appearance abruptly when the animals had been infected 2 months and, after new cases had developed for a period of about 10 days, it disappeared equally as abruptly. In considering the nature of the condition the most obvious possibility seemed to be that we were dealing with a tuberculous meningitis. The symptoms observed in our guinea pigs bore a close resemblance to those of experimental tuberculous meningitis of rabbits as described by Austrian (1), Kasahara (2) and Soper and Dworski (3) and of dogs as described by Manwaring (4). However, the occurrence of the malady in such a high proportion of animals, together with its abrupt onset and disappearance, made it seem essential to determine whether we were not dealing perhaps with some superimposed infectious disease of the central nervous system. Conceivably tubercle bacilli might be present and yet not be the essential cause of the paralytic condition. Because of this possibility it seemed advisable to maintain the paralytic disease for study by serial passage in guinea pigs. This was successfully accomplished by the intracerebral injection of emulsions of brain from animals showing the paralysis.

EXPERIMENTAL

There were three evident possibilities to consider in connection with the paralytic disease besides the tubercle bacillus. The first was infection with the filterable virus causing Roemer's (5) guinea pig paralysis; for the disease we were studying exhibited many points of similarity with that malady. A second possibility was that the animals had been infected with a low grade herpes virus at the time of inoculation, since the culture of tubercle bacilli used in this infection had been but quite recently isolated from human sputum. Third, the condition might have been an infection of the central nervous system by some visible organism other than the tubercle bacillus. The investigation was conducted with due reference to the points thus brought up.

The general plan followed in passing the paralytic condition from one animal to another was to remove the brain, under aseptic precautions, from a paralyzed guinea pig and after preparing an approximately 10 per cent emulsion of it in physiological salt solution to inject 0.1 cc. of the emulsion intracerebrally into a trephined normal animal.* The disease was maintained in this way through 9 successive groups of guinea pigs; and the clinical picture remained in all essential details the same throughout. The time elapsing between inoculation and the appearance of the first symptoms of illness varied from 8 to 27 days; and the tendency was for this period to shorten with each successive passage. The incubation period was remarkably constant for the animals of each passage group, however.

The disease as observed in guinea pigs infected experimentally was very uniform in its manifestations. For a period of 12 to 24 hours following the inoculation the animals appeared somewhat listless and disturbed, doubtless because of the procedure to which they had been submitted. Thereafter throughout the incubation period they remained bright, active and normal. The first symptoms noticed were a recurrence of listlessness and some roughening of the fur. The animal sat quietly in its cage when not disturbed. There was usually some faulty coordination and the guinea pig had difficulty in getting to its feet when placed suddenly on the back. Very soon the incoordination became more marked. It was usually most noticeable in the hind quarters. Other symptoms referable to the central nervous system that were very frequently seen at this time were hyperesthesia, a tendency to move in circles, marked tremor, especially of the head, and a tendency to torticollis. A few animals showed a marked nystagmus. As a rule, the day following the onset of these symptoms a definite weakness of the hind quarters was evident, and there was often loss of sphincter control of both bladder and

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* Ether anesthesia was practiced for all intracerebral inoculations.

rectum. This was followed very soon by a definite posterior paralysis, spastic at first and later flaccid. Very occasionally a paralysis of one or both fore legs preceded the posterior paralysis, and again, rarely, the condition resembled hemiplegia more than paraplegia. Following the early period of listlessness the animal appeared bright, and alert even when there was a complete posterior paralysis. One received the impression that the systemic reaction to the infection was very slight. Fig. 1 clearly illustrates the animals' state. As a rule, after 3 to 4 days of paralysis, its general condition suddenly became worse, it lost consciousness, the fur roughened, and, usually within 24 hours, death ensued. In 23 guinea pigs the disease was allowed to progress to a fatal termination and the average survival of these following the onset of the symptoms was 5.7 days, the extremes being 1 and 11 days. We had 6 animals die very suddenly, at the termination of

FIG. 1. Guinea Pig Y of the 7th serial passage showing complete posterior paralysis.

what corresponded to the incubation period in other animals of the same inoculation group, without having shown any symptoms whatsoever. No animal in our series that was inoculated with a fresh brain emulsion from either a "spontaneous" or experimental case was immune to infection. In animals living longer than 5 days after onset of the paralysis much loss of weight occurred. It took place, however, to some extent, in all of the animals, beginning late in the period of incubation. In a few, the onset of symptoms referable to the central nervous system was preceded by a very evident loss of weight. At no time was there any febrile reaction to the infection, although when the animal became moribund, the temperature occasionally became subnormal.

Pathology.—In animals in which the condition had been induced by the intra-cerebral injection of brain emulsion from a previous case a few macroscopic changes were consistently found at autopsy. On opening the calvarium an excess of cerebrospinal fluid was always encountered. This was usually slightly turbid. Nothing abnormal was evident in the brain itself, nor was there anything charac-teristic along the needle track. Rarely the meninges were adherent to the skull at the trephine region. They were usually somewhat thickened over the area of the mid-brain, pons and medulla at the base of the skull. Examination of the cord revealed no gross lesions.

Outside the central nervous system the only evidence of pathological altera-tion was found in the spleen. This was usually somewhat larger than normal and was studded with hyperplastic follicles.

Microscopic examination of paraffin sections of brain stained by the usual methods showed a great abundance of acid-fast bacilli in the thickened pia mater but none elsewhere. The condition was found to be a true meningitis with some extension into the encephalon along the lines of the larger blood vessels but else-where the encephalon was singularly free from infiltration or other lesions. The pia mater, especially at the base of the brain, was definitely, but not extremely, thickened. The exudate consisted of mononuclear cells, some of them lympho-cytes, but large mononuclears chiefly. Necroses were lacking and giant cells and definite tubercle formation were not seen.

Bacteriology.—The brain emulsions as prepared for inoculation were examined for tubercle bacilli and other organisms. In the first of these emulsions to be studied tubercle bacilli were easily demonstrated by the Gabbet method, but in the material similarly prepared from later passages this was no longer possible. Many of the examinations were casual but some were prolonged and thorough.

Cultures of these brain emulsions were made on various media. No growth was obtained in any case on plain or blood agar. On Dorset's egg medium growth of typical tubercle bacilli was quite regularly obtained and these even from speci-mens in which no tubercle bacilli had been demonstrable in stained smear prepara-tions. Cultures of brain emulsion in a modified Noguchi Leptospira medium (guinea pig serum was substituted for rabbit serum) showed an opalescent clouding of the upper portion of the medium after incubation at 37.5°C. for 10 days to 2 weeks. Later a granular sediment appeared. The upper growth was found on examination to be a pleomorphic Gram negative bacillus. It was non-acid fast by the ordinary Ziehl-Neelsen technique, but when decolorized after the method of Gabbet showed some acid-fast forms. The granular sediment was entirely non-acid-fast. Such cultures were recovered from nine different guinea pigs,— all that were so cultured. After 3 passages on media, guinea pigs were inoculated intracerebrally, some with the upper, diffuse growth and others with the sediment-ary growth from one of these cultures. All developed the typical posterior paraly-sis with its accompanying symptoms. Animals inoculated subcutaneously with the upper portion of the culture developed within 4 days nodules the size of a

split pea at the site of inoculation. This was followed very soon by the appearance of large, shotty, inguinal lymph nodes. The nodules at the site of inoculation were discharging a cheesy pus within 2 weeks, and numerous organisms similar to those that had been grown in Noguchi media were found in this caseous material. Autopsy of these animals 2 months later revealed the presence of lesions typically encountered in guinea pig tuberculosis. The organism isolated and cultured in Noguchi medium was obviously a tubercle bacillus despite its atypical staining reactions.

Susceptibility of Rabbits.—Recourse was had to inoculations into rabbits in order to determine whether we were dealing with a mild form of herpes encephalitis, or with Roemer's virus which is described as specific for guinea pigs. Two rabbits were inoculated intracerebrally with brain emulsion from guinea pigs of the 3rd and 7th experimental serial passages. The animals developed a typical posterior paralysis with loss of bladder and rectal sphincter control, after incubation periods of 25 and 39 days respectively. There was nothing in their disease to suggest herpes encephalitis. Brain emulsions from these animals injected intracerebrally produced the disease in a second series of rabbits.

Non-Filtrability of the Infectious Agent.—Five Berkefeld N filtrates of brain emulsions from experimentally infected guinea pigs have failed to produce the disease when injected intracerebrally into 16 guinea pigs and 1 rabbit. Three of these filtrates were prepared by merely grinding the brain with sterile sand and salt solution in a mortar and then filtering; one was prepared by grinding in the same way, centrifuging the material, and passing it through cotton and paper before filtration; and in one, after the grinding, the preparation was shaken for half an hour with sand and glass beads and filtered first through cotton and paper and then through a Berkefeld N filter.

DISCUSSION AND SUMMARY

The experimental data collected during this study of a transmissible type of paralysis developing in tuberculous guinea pigs indicate the condition to be a true tuberculous meningitis. We have been able to rule out the possibility that it is due to a non-tuberculous infection of the central nervous system caused by Roemer's virus, or by an atypical herpes virus, or by some bacterium other than the tubercle bacillus. Roemer's virus and herpes could be eliminated from considera-

tion when Berkefeld N filtrates of infectious brain emulsions proved incapable of reproducing the disease. Furthermore, rabbits could be infected as they cannot with Roemer's virus, and the disease elicited in rabbits bears no semblance to herpes encephalitis. No organism other than the tubercle bacillus could be obtained on culturing brain or brain emulsions from experimental cases, and no others were seen in examining fresh smear preparations from the central nervous system. In a modified Noguchi medium a tubercle bacillus possessing atypical staining properties was obtained. This organism was capable of producing the typical paralytic disease when injected intracerebrally into guinea pigs, and also generalized tuberculosis in animals inoculated subcutaneously with it.

Typical tubercle bacilli were readily demonstrable in sections of the meninges from animals with the disease, and culture of pieces of brain on Dorset's egg medium usually yielded a growth of tubercle bacilli. Only in the first of the experimental passages, on the other hand, was it possible to demonstrate acid-fast organisms in fresh smear preparations from the central nervous system. This fact and the attributes of the atypically staining organisms encountered in the cultures in Noguchi media will be considered more fully in a subsequent publication.

In view of the much discussed question of the filtrability of the tubercle bacillus our observations concerning the failure of this organism to pass a Berkefeld N filter are of interest. No animal in our series inoculated intracerebrally with brain emulsion from either a "spontaneous" or experimental case of tuberculous meningitis failed to develop meningitis, and that rather acutely, while no animal in our series injected with a Berkefeld filtrate of brain emulsion has developed tuberculous meningitis or any other form of tuberculosis. In connection with this observation it must be recalled that the organism was atypical in respect to its staining qualities at least.

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THE CULTURAL AND STAINING REACTIONS OF A STRAIN OF THE TUBERCLE BACILLUS PRODUCING PARALYSIS IN GUINEA PIGS

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In a preceding paper by Shope and Lewis (1) account is given of a transmissible paralytic condition arising in tuberculous guinea pigs. It was believed possible that it had resulted from secondary infection with some neurotropic "virus." The methods of study adopted were designed primarily to establish or discredit this conception. The departures from the conventional technique so long rigidly adhered to by students of the tuberculosis problem have given rise to unexpected and significant results, one aspect of which it is the purpose of this paper to develop.

OBSERVATIONAL

The culture of the tubercle bacillus with which we are here concerned (Designated P. J.) was isolated in the summer of 1927 from the sputum of a colored adult male with chronic pulmonary tuberculosis, in the tuberculosis wards of the Philadelphia General Hospital, Philadelphia, Pa. The sputum showed many acid-fast bacilli when stained by Gabbet's method. Some of it about 24 hours old was injected subcutaneously into a number of guinea pigs, and these developed generalized tuberculosis in the typical manner. Four to six weeks after inoculation cultures were made on Dorset's egg medium from the spleens of several of these animals. No contaminations were encountered. The cultures developed rapidly and vigorously for the most part. After six weeks growth a representative tube was selected and transfers were made to Dorset's egg medium. After another eight weeks the second series of tubes were used for the inoculation of the large series of guinea pigs referred to in a preceding paper (1). The disease developed characteristically in them, except for the unusual occurrence of paralysis in some of the animals. The general virulence of the culture as estimated by the average length of life of the large series of animals was neither especially high nor low.

The culture P. J. has been maintained by rather infrequent transfers on Dorset's

egg medium and presents typical appearances thereon. The culture is acid-fast except that after decolorization by 30 per cent nitric acid followed by alcohol, counterstaining with Loeffler's methylene showed a few blue staining rods. It has not up to now been completely tested in all respects but there seems to be no reason to question the fact that the strain is a tubercle bacillus of human type.

When cases of paralysis appeared among the guinea pigs inoculated with the culture a successful effort was made to transfer the paralytic condition to other guinea pigs by direct intracerebral inoculation with brain emulsion from a case of the disease. This series of inoculations, from guinea pig to guinea pig by the intracerebral route, has been continued up to the present time. In the first emulsions made of brain and cord tubercle bacilli were easily demonstrated by the Gabbet method. But in the material similarly prepared from later passages this was no longer possible. Many of these examinations were casual but some were prolonged and thorough. Paraffin sections stained by the usual methods showed a great abundance of acid-fast bacilli in the thickened pia mater but none elsewhere.

Cultures of brain emulsions were made on various media and on Dorset's egg medium a typical growth of tubercle bacilli could usually be obtained. Those cultures of interest to the present discussion were made on a medium that is a variant of the one recommended by Noguchi for the cultivation of Leptospira. Ordinary nutrient dextrose agar was diluted 1 + 8 with distilled water, the diluted agar was boiled, cooled to 50°C., and 10 per cent of sterile guinea pig serum was added. Thorough mixture was effected at this temperature and the medium tubed in long narrow culture tubes (200 x 13 mm.). Portions of various of the emulsions of the nervous tissues were planted in these tubes, using a long capillary pipette in such a way as to form a thin streak from the bottom of the tube to the top along one side near the glass, with a small extra deposit on the upper surface of the semisolid medium. These plants were incubated at 37.5°C. in a moist thermostat for several months.

Certain of these tubes showed rapid growth of bacteria no two strains of which were alike and these were soon discarded. The others gradually showed the development of a faint cloud in the upper 10 mm. of the medium. This eventually became coarsely granular. It could be transferred to other tubes of the same medium and developed the same appearance. Exceptional tubes showed a faint haze toward the bottom with a clear intermediate zone.

When coverslip or slide preparations of the growth were fixed with methyl alcohol and stained with Loeffler's methylene blue, using heat to the point of steaming, and the staining process was continued for 3 to 5 minutes, rather definite, faintly staining, thin, small bacilli were found in increasing numbers as growth progressed. Stained by Gram's method in the usual way, employing Stirling's gentian violet, the bacilli were always Gram negative. The counterstains demonstrated them but faintly and it was frequently necessary to restain the preparation with Loeffler's methylene blue as above described to be sure of the presence of the

bacilli in the preparation.   Such cultures were recovered from nine different guinea pigs,—all that were so cultured.

As stated in the preceding paper, the paralytic syndrome was readily reproduced by the intracerebral injection of these cultures in several instances.   When culture material was introduced subcutaneously local lesions and lesions of the adjacent lymph nodes were produced, and autopsy of these animals two months later revealed the presence of lesions typical of guinea pig tuberculosis.   This made it appear likely that the organism grown in Noguchi medium was a tubercle bacillus.   Otherwise it was necessary to postulate the presence of a microörganism capable of inducing a disease simulating tuberculosis very closely.   This question was now taken up directly.

When the cultures in the semisolid medium were restudied the following facts were made clear:

The bacilli however fixed and no matter how long and intensely stained with carbolfuchsin were always decolorized by 30 per cent nitric acid followed by alcohol, or by 3 per cent hydrochloric acid in 70 per cent alcohol; though it was indeed possible to make the staining so persistent that the background of culture material also retained a muddy red color after the application of acid, and then sometimes bacilli of the same dubious color could be made out with difficulty. The typical bright red of the Ziehl-Neelsen stain was never seen.

If the preparations were fixed with heat and stained by Gabbet's method a considerable number of bacilli retained the stain.   If stained by Gabbet's method after fixation with methyl alcohol usually none of the bacilli retained the fuchsin stain but sometimes a few did.   A few blue-staining bacilli were also usually seen. If the heat fixed or methyl-alcohol-fixed Gabbet stained preparations were further stained with Loeffler's methylene blue many blue stained bacilli were found.   If the preparations were fixed in 4 per cent formaldehyde for 3 hours or more the number of bacilli retaining the fuchsin stain after Gabbet's methylene blue was often, but not always, greater than when fixation was by either heat or methyl alcohol.

Taking the matter from a slightly different standpoint: if the heat fixed preparations were stained with carbolfuchsin in the usual manner and decolorized by acid and alcohol separately it was found that 30 per cent nitric acid and 20 per cent sulfuric acid decolorized many but not all of the bacilli.   The slightest application of alcohol, however, after these strengths of mineral acid removed all the stain.   Even though much weaker mineral acids were used—5 per cent sulfuric, 3 per cent of either nitric or hydrochloric—alcohol later removed all the stain.

If Gram stain was employed according to the general plan of the Much method, with some modifications, the bacilli retained the stain.   The essential feature of

this method is that the gentian violet is phenolized (2 per cent) and the stain when applied is heated. The iodin treatment is also prolonged and decolorization is done with equal parts of acetone and absolute alcohol. Under these conditions all of the bacilli retain the Gram stain. Every effort to show that some were Gram positive, others Ziehl-Neelsen positive has failed.

DISCUSSION

The preceding observations present two points of considerable interest with regard to the tuberculosis problem. The first relates to the identity of the culture and the criteria on the basis of which any microörganism is to be identified as a true tubercle bacillus. The second relates to the conditions of growth of the bacillus and their effect on its staining reactions.

The methods for identifying the tubercle bacillus as they now stand have been developed primarily in the interests of microscopic, clinical diagnosis. It has become a dogma in America (2) that if a bacterium will not, after staining with carbolfuchsin, withstand decolorization by 30 per cent nitric acid followed by alcohol it is not a tubercle bacillus. Heat fixation is always recommended for smear preparations but nothing is said as to the availability of other fixing methods. It is to be assumed however, since alcohol, formaldehyde, or chromium fixatives are more or less indiscriminately used for tissue work, that these are to be considered adequate for the demonstration of tubercle bacilli in smear preparations as well.

In Germany a similar dogmatic attitude is taken with reference to the critical value of 3 per cent hydrochloric acid in 70 per cent alcohol (acid alcohol) (3). To qualify as a tubercle bacillus a bacterium must resist decolorization by this agency. Our culture meets the classical requirements to the extent that it produces disease in animals of characteristic sort, that the bacilli in the fixed tissues resist decolorization, and the cultures on Dorset's egg medium present typical characteristics.

All this being true, it is astonishing to find that the smear preparations of the bacteria from the nervous system of the animals we have studied, when fixed in methyl alcohol, resist decolorization so little that their presence can pass unrecognized even though the weaker Gabbet method is employed for their demonstration, and that the culture in the semisolid medium yields organisms at wide variance

with the classical requirements.   We are concerned with a bacterium which is moderately acid-fast, not alcohol-fast at all, and in which the particular method of fixation influences the result in ways not previously recognized.   We are led by these observations to the conclusion that Much (4), and those who have followed him, are right in their contention that the tubercle bacillus may at times be positive to Gram stain and demonstrable by means of it, yet negative to the Ziehl-Neelsen method rigidly applied.*   The weakness of Much's contention has in large measure been that the conditions under which such forms appear have never been accurately defined.   Our observations when pushed further may supply this deficiency.

That the tubercle bacillus should be readily and easily grown in the semisolid medium of Noguchi is somewhat surprising, certainly interesting, and possibly useful.   If other cultures, and cultures from other organs behave like the present one, the method would be the simplest and most certain way to recover the organism from the infected animal. Whether the virulence or other qualities of the microörganism are affected has yet to be determined.   The medium contains no glycerine and hence differs from the other media recommended for the cultivation of the tubercle bacillus, with the exception of the coagulated egg of Dorset or its modifications from which the glycerine is sometimes omitted.

CONCLUSIONS

When a strain of human tuberculosis was carried from guinea pig to guinea pig by intracerebral inoculation the bacillus of tuberculosis could be readily recovered in the semisolid "Leptospira" medium of Noguchi simply modified by the substitution of guinea pig for rabbit serum.   Whether even this modification is required remains to be determined.

The tubercle bacillus in these cultures and possibly in the nervous tissue as well differed from the classical type in that it was less acid-fast and quite lacking in alcohol fastness.   However, it retained the Gram

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* These observations have no bearing whatever on the further contention of the same school that there is a non-bacillary or granular form of the tubercle bacillus.   Thus far, the bacillary form only has appeared in our animals and cultures.

stain as modified by Much. Staining reactions, as this culture of the tubercle bacillus shows, are not absolute qualities but are variable with conditions, a consideration which it may be important to recognize. Efforts to define these conditions more precisely are likely to be productive.

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THE REMOVAL OF AGGLUTININ FROM SENSITIZED MOTILE BACTERIA

SECOND PAPER. THE AGGLUTINATIVE PROPERTIES OF WATER WASHINGS

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Observations on the removal of agglutinin from sensitized motile bacteria were presented and discussed in a preceding paper.[1] Extraction of the sensitized test organism (*Bacillus aertrycke*) in a 5 per cent sodium chloride solution at a temperature of 60°C. resulted in the removal of considerable flagellar agglutinin while the somatic agglutinin was practically unaffected. Brief mention was made of an anomalous reaction which occurred when distilled water was employed as the extracting or washing medium. The reaction is described in detail in the following experiments together with some additional observations leading to a possible explanation.

General Methods

A normal actively motile strain of *B. aertrycke* (guinea pig paratyphi, Type II) recently isolated from the spleen of a naturally infected guinea pig was used as the test organism. Cultivation was carried out on moist Blake bottles, inoculated with a sufficient amount of young broth culture to cover the surface of the agar and incubated at 37°C. for 18 hours. Cultivation on a dry medium lowers the floccular agglutinability of the bacteria. The growth was removed with normal saline, transferred to a graduated centrifuge tube, and sedimented for an hour. The supernatant was discarded and the bacteria, still in a packed state, were rinsed once with saline.

The serum was from a rabbit immunized with the above strain of *B. aertrycke* and it contained both flagellar and somatic agglutinins. For sensitization of the bacteria the following materials were used: 0.5 cc. of packed bacteria, 0.1 cc. of

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[1] Nelson, John B., *J. Exp. Med.*, 1928, 48, 825.

antiserum, 2.4 cc. of saline. This formula gives the minimum proportion of packed bacteria required for the nearly complete removal of flagellar agglutinin with the serum dilution employed. It was about twice the amount required in the preceding work[1] with a different strain of *B. aertrycke* and its homologous antiserum of approximately the same titer. The bacteria were resuspended and the mixture incubated at 37°C. for 5 hours followed by overnight refrigeration.

The mixture was now centrifuged for 1 hour at the same speed and the supernatant removed. The packed sensitized bacteria were suspended in 2.5 cc. of distilled water and again centrifuged for an hour. The washing process was continued 3 to 5 times with fresh water after each sedimentation.

The agglutinin content of the absorbed antiserum and of the washings was determined by macroscopic agglutination with 0.5 cc. of antigen and 0.5 cc. of fluid dilutions. As a control, unabsorbed antiserum diluted and incubated similarly to the absorbed serum was tested in the same way. Two antigens designated whole and heated were used. The former was a fresh saline suspension of *B. aertrycke* and contained both flagellar and somatic components. The latter was a saline suspension heated to 100°C. for 30 minutes, washed, and resuspended in saline. It contained only the somatic component. Both suspensions were standardized to 2.4 with the Gates apparatus. Incubation was carried out at 37°C. for 3 hours followed by overnight refrigeration.

Experiments

The results of a typical experiment with the use of distilled water as the medium for the removal of agglutinin are given in Table I. The agglutinin titer of the absorbed antiserum mixture and of the several water washings is compared with that of the unabsorbed antiserum. If we take into account the initial dilution of the unabsorbed and absorbed antiserums, the actual titers are 25 times the figure given.

The unabsorbed antiserum agglutinated the whole antigen in high dilution. The type of clump was mixed, though predominantly flocular in the lower dilutions, while in the higher dilutions it was purely floccular. It agglutinated the heated or deflagellated antigen in the lower dilutions only with a purely granular type of clump. While the limit of agglutination for the whole antigen was identical with that of the antiserum used in the preceding work,[1] the intensity of the reaction in the lower dilutions was less with the present antiserum. The limit of agglutination for the heated antigen was one dilution higher with the earlier antiserum, and the intensity of the reaction was likewise more marked. The absorbed antiserum gave a slight, floccular agglu-

tination in very low dilution with whole antigen but no reaction with heated antigen the lowest dilution possible. The single absorption afforded an approximately 99 per cent removal of both flagellar and somatic agglutinins.

TABLE I

Agglutination of B. aertrycke by Unabsorbed Antiserum, Absorbed Antiserum and the Water Washings of a Sensitized Suspension

Test fluid	Antigen	Test fluid dilutions										
		2	4	8	16	32	64	128	256	512	1,024	2,048
Unab- sorbed serum	W*	++++ M	++++	++++	++++	+++	+++	+++	++	++	+	—
	H	++++ G	+++	++	+	±	±	—	—	—	—	—
Absorb- ed serum	W	+ F	±	trace	—	—	—	—	—			
	H	—	—	—	—	—	—	—	—			
1st water washing	W	± F	trace	—	—	—	—	—	—			
	H	—	—	—	—	—	—	—	—			
2nd water washing	W	++ F	++	+	+	+	±	±	—			
	H	++ F	+	±	±	±	—	—	—			
3rd water washing	W	++ F	++	+	+	±	±	—	—			
	H	++ F	+	±	±	—	—	—	—			
4th water washing	W	++ F	+	+	±	±	—	—	—			
	H	+ F	±	±	±	—	—	—	—			

* W = whole bacteria, H = heated bacteria (100°C.), M = mixed agglutination, F = floccular agglutination, G = granular agglutination. The same abbreviations are used in all the subsequent tables.

With the first water washing there was a slight floccular agglutination in the presence of whole antigen only. The following washings reacted quite differently. With the second washing there was a marked increase in both the intensity and the titer limit of agglutination with the whole antigen. The type of clumping was floccular. In addition there was a reduced but perfectly definite agglutination

in the presence of heated antigen. The type of clump was likewise floccular, with this difference that the aggregates were more loosely formed and became less compact upon standing than the usual floccular clumps. The subsequent washings duplicated the reaction though on an increasingly lower level.

When the above reaction is viewed as it stands, it appears that the suspension of sensitized motile bacteria in a salt-free medium results in the removal of agglutinin which gives a floccular type of agglutination not only in the presence of bacteria with their flagella attached but also in the presence of deflagellated bacteria. The occurrence of a floccular agglutination in the presence of deflagellated bacteria is contrary to the many observations which have been made on the agglutinative behavior of motile organisms.

Repetition of the above experiment with other strains of the same bacterial species and with another species of Salmonella (*B. paratyphi,* Type I), likewise of guinea pig origin, gave essentially similar results. The agglutinin titer of the second water washing fluctuated between 1:64 and 1:256 with a corresponding fluctuation in the titer of the other washings. In some cases the second and third washings were of equal titer. It may be noted that the intensity of the agglutination reaction with the water washings was regularly less marked than that of the extraction fluids in the preceding work.[1]

Upon consideration of the reaction it seemed possible that the flocculation which occurred in the presence of heated or deflagellated antigen might be due to the precipitation of material present in the washings. These, with the exception of the first, regularly showed a distinct turbidity which swirled visibly upon agitation. It was not due to bacteria, although the washings was never entirely free of bacteria. There was no sedimentation upon standing. The degree of turbidity tended to decrease with successive washings but a gradation was not always apparent. The stability of the washings in the presence of salt was determined by serial dilution with saline.

Sensitized bacteria were washed four times with distilled water and a portion of each washing heated to 70°C. for 30 minutes. The unheated and heated washings, in a total volume of 1 cc., were diluted serially with saline and incubated without the addition of antigen. The findings with the antigen-free washings are given in Table II.

There was no reaction with the first unheated washing. The others showed a graded flocculation with the settling out of a loose fluffy sediment which was easily dispersed upon agitation. There was never any flocculation with the heated washings. In appearance and amount the sediment was identical with that of the washings in the presence of heated antigen and in appearance with that of a pure flagellar suspension in the presence of specific antiserum. The material present in the washings likewise resembled flagella in its thermolability. Centrifuged suspensions of flagella, however, unlike the water wash-

TABLE II

The Reaction of Unheated and Heated Water Washings of a Sensitized
Suspension upon Dilution with Normal Saline

Test fluid	State	Test fluid dilutions					
		2	4	8	16	32	64
1st water washing	Unheated	—	—	—	—	—	—
	Heated	—	—	—	—	—	—
2nd water washing	Unheated	+ + F	+	±	±	±	—
	Heated	—	—	—	—	—	—
3rd water washing	Unheated	+ + F	+	±	±	±	—
	Heated	—	—	—	—	—	—
4th water washing	Unheated	+ F	±	±	±	—	—
	Heated	—	—	—	—	—	—

ings, are nearly water clear. The presence of salt-precipitable material in all the washings save the first was definitely indicated.

The water washings always gave a more marked agglutination, both as to titer and to intensity, in the presence of whole antigen than in the presence of heated antigen. The reaction with the whole antigen was regarded as essentially a true agglutination due to flagellar agglutinin removed from the sensitized bacteria by the washing process. Since flagellar agglutinin is heat-stable at 70°C., at which temperature the washings no longer flocculate with saline alone, it was possible to test this assumption by heat inactivation.

Two series of 3 water washings from sensitized bacteria were em
ployed. One series was heated at 70°C. for 30 minutes. The oth
was used unheated. Both were tested as usual against whole an
heated antigen. The reactions with the heated washings are given .
Table III. The unheated washings behaved essentially as befo:
(Table I) and the results are not included.

The heated washings agglutinated the whole bacteria with the usu
floccular clumping which was more compact than that of the typic
clumping with pure flagella. As to titer, there was little differen

TABLE III

The Agglutination of B. aertrycke by the Heated Water Washings of a Sensitized Suspension

Test fluid	Antigen	Test fluid dilutions							
		4	8	16	32	64	128	256	512
1st water washing	W	++ F	±	—	—	—	—	—	—
	H	—	—	—	—	—	—	—	—
2nd water washing	W	++ F	++	++	+	+	trace	—	—
	H	—	—	—	—	—	—	—	—
3rd water washing	W	++ F	++	++	+	+	trace	—	—
	H	—	—	—	—	—	—	—	—

between the unheated and heated washings. Unlike the unheate
washings, however, the heated ones failed to react in the presence c
deflagellated antigen. It seems evident that the floccular reactio
with whole antigen was a true agglutination due to the presence c
flagellar agglutinin in the washings. That the water washings fror
the sensitized bacteria, with the exception of the first, containe
flagella in suspension was a suggestive fact. The actual presence c
flagella, under the conditions of the experiment, was difficult to demon
strate. Some observations, however, on the behavior of unsensitize
motile bacteria are presented as contributing evidence.

0.5 cc. amounts of *B. aertrycke*, in centrifuge tubes, were resuspended with 2.
cc. of distilled water and sedimented. Because of the difficulty in getting clea
supernatants with unsensitized bacteria the time was increased to 90 minutes an

the speed from approximately 2,000 to 2,500 R.P.M. The bacteria were washed four times in this way. While the washings were not quite water-clear they failed to show the characteristic turbidity of the washings from sensitized bacteria. One lot was diluted serially with saline in a total volume of 1.0 cc. and incubated without antigen for the usual length of time. The other lot was diluted serially

TABLE IV

The Reaction of Unheated Water Washings of Unsensitized Bacteria upon the Addition of Antiserum and of Normal Saline

Test fluid	Dilution fluid	Test fluid dilutions						
		2	4	8	16	32	64	128
1st water washing	Immune serum Saline	++ F —	+ —	+ —	± —	± —	± —	— —
2nd water washing	Immune serum Saline	++ F —	+ —	± —	± —	± —	— —	— —
3rd water washing	Immune serum Saline	+ F —	+ —	± —	± —	— —	— —	— —
4th water washing	Immune serum Saline	+ F —	± —	± —	— —	— —	— —	— —

TABLE V

The Agglutinability of Whole and Washed Suspensions of B. aertrycke

Antigen	Serum dilutions									
	4	8	16	32	64	128	256	512	1,024	2,048
Whole bacteria	++++ M	++++	++++	+++	+++	++	++	++	+	—
Washed bacteria	++++ M	++++	+++	++	+	±	trace	trace	—	—

with saline in a volume of 0.5 cc., and 0.5 cc. of a 1:400 dilution of homologous antiserum added to each lot. Incubation was carried out in the same way. The washed unsensitized bacteria were standardized in saline and their agglutinability compared with that of unwashed whole bacteria. The same antiserum, used throughout, was employed. The observations on unsensitized bacteria are presented in Tables IV and V.

The washings from the unsensitized bacteria failed to flocculate upon the addition of saline. When dilute antiserum was added, however, flocculation occurred and the type of clumping was identical with that of the water washings from sensitized bacteria in the presence of saline. It was a typical flagellar agglutination. Unlike the series from sensitized bacteria, however, the initial washing contained flocculable material. That the bacteria present in these washings were too few in number to influence the reaction was shown by the following experiment.

Sedimented whole bacteria were rinsed and resuspended in saline. The suspension contained only bacteria; there were no free flagella. The suspension was then diluted with saline to a barely visible turbidity. A 0.5 cc. portion of this suspension was carried through five serial dilutions and 0.5 cc. of a 1:400 dilution of antiserum added to each tube. There was no agglutination in any dilution after the usual incubation.

The agglutinability of the washed unsensitized bacteria was considerably less than that of unwashed bacteria. As shown in Table V, the titer was decreased and the intensity of the reaction outside the zone of granular agglutination was markedly less with the former. The above observations point to the removal of a certain number of flagella from the bacteria with each washing manipulation. Upon centrifuging, the bacteria are sedimented while the flagella remain free in the supernatant. With the addition of specific antiserum flagella and agglutinin unite and a floccular agglutination results.

DISCUSSION

The preceding observations have proved readily reproducible with the technic outlined. An interpretation linking them together and offering a possible explanation of the described anomaly is presented:—

The resuspension of sedimented motile bacteria in a fluid medium results in the mechanical removal of some of the attached flagella. If the bacteria are unsensitized the flagella remain in suspension after centrifugation, and the clear supernatant gives a typical flagellar agglutination upon the addition of specific antiserum. If the bacteria are sensitized the free flagella, which are in combination with agglutinin, clump in the presence of salt and are removed upon centrifugation. The initial water washing of sensitized motile bacteria contains suffi-

cient salt from the previous absorption mixture to cause clumping of the flagella. The clumped flagella are removed by sedimentation and consequently the supernatant is inactive, failing to flocculate upon the addition of saline. The subsequent water washings are salt-free and the freed flagella, either with attached or freed agglutinin, remain in suspension. The water washings of sensitized bacteria are cloudy at this stage owing possibly to minute aggregates of flagella which are too small for sedimentation.

That agglutinin in some form is removed from flagella in the salt-free medium seems apparent. Nothing definite can be said concerning its physical state other than the fact that it is again able to unite with whole bacteria, causing a clear-cut agglutination in moderate dilution.

The water washings of sensitized bacteria, at this stage, also flocculate upon the addition of saline alone. The flocculation is in reality a flagellar agglutination which was previously inhibited by the absence of salt. In the presence of salt, recombination of flagella and agglutinin, or union of flagella already in combination with agglutinin, occurs, with subsequent clumping.

It is maintained that the floccular reaction which results when the salt-free water washings are mixed with a saline suspension of heated bacteria is in reality a flagellar agglutination and is entirely independent of the added antigen. Flagella and flagellar agglutinin are both present in the salt-free washing fluids. Salt is supplied by the added bacterial suspension (heated) and flagellar agglutination results. In the presence of whole bacteria agglutinin may be diverted from the free flagella by the excess of those attached to the bacteria causing under such circumstances a bacterial agglutination.

As regards the differential removal of agglutinin it may be said that flagellar agglutinin in a state capable of again causing flocculation of whole bacteria may be freed from sensitized motile bacteria by washing with salt-free water. With the degree of sensitization brought about in the work here reported somatic agglutinin, on the other hand, is not demonstrable in the washings.

SUMMARY

The salt-free water washings of a sensitized motile bacterium (*B. aertrycke*) were found to cause a floccular agglutination in the

presence of both whole and deflagellated antigen. Evidence was presented that the water washings when salt-free contained flagella and flagellar agglutinin and that clumping occurred upon the addition of saline. The floccular reaction in the presence of deflagellated bacteria was regarded as the agglutination of flagella present in the washings. In the presence of whole bacteria, however, actual bacterial agglutination resulted.

The writer is indebted to Dr. John H. Northrop for helpful criticism during the course of the work.

THE EFFECTS OF LOSS OF GASTRIC AND PANCREATIC SECRETIONS AND THE METHODS FOR RESTORATION OF NORMAL CONDITIONS IN THE BODY

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From careful experimental (1, 2, 3) and clinical (4) studies, it has become well recognized that continued loss of gastric juice may lead rapidly to severe or fatal dehydration and alkalosis, due to secretion into the stomach with subsequent loss of large amounts of water and chlorine ion, together with a considerably smaller, but still significant amount of fixed base. Theoretically the most effective method of relieving such changes should be the intravenous administration of adequate amounts of a solution of the same inorganic composition as gastric juice, i.e. a solution in water of HCl and $NaCl$ in 100 mM and 30 mM concentrations respectively. Practically, however, administration of 145 mM or .85% $NaCl$ (physiological salt solution) in sufficient quantity is quite effective. This is due largely to normal renal activity which permits excretion of excess of fixed base in the form of $BHCO_3$ during the period after the normal fixed base level has been restored and while the normal Cl concentration has not yet been reached. The general use of salt solution in such cases, therefore, is well justified from both the theoretical and practical viewpoint.

In certain conditions, however, which include loss of combined gastrointestinal secretions, as for instance intestinal obstruction or fistula, and severe diarrhea, dehydration may be accompanied by less marked alkalosis, or even by severe acidosis, due presumably to greater loss of fixed base than fixed acid in the unabsorbed digestive secretions, together with the accumulation of such acids as lactic, phosphoric, sulfuric and occasionally ketone, dependent upon inadequate circula-

tion, urinary excretion and carbohydrate metabolism.   Theoretically the most effective method of relieving such changes should be parenteral administration of adequate amounts of water, base, bicarbonate and chloride (together with dextrose in the case of ketosis) in the proportions in which they exist in the gastrointestinal secretions lost. Difficulties arise, however, in applying such treatment because our knowledge as to the normal proportion of water, fixed base and fixed acid in the combined intestinal secretions is not as complete as in the case of gastric juice, and because of the difficulty of preparing sterile and non-irritating solutions containing sufficient amounts of all the substances lost.   Practically, however, excellent relief of dehydration and acidosis has frequently been seen following the administration of large amounts of saline or Ringer's solution, with or without the addition of dextrose, the reason again being normal renal activity which permits, in this instance, excretion of the excess of fixed acid (Cl^-) in combination with manufactured base (NH_4^+) with resultant retention of fixed base (B^+) for combination with HCO_3.   However, in many instances of severe diarrhea associated with persistent oliguria (5) as well as in cases of nephritis (6) Hartmann noted that the administration of large amounts of Ringer's solution was not followed by adequate restoration of $BHCO_3$.   By following closely the chemical changes taking place in the blood plasma of such cases and administering Ringer's solution, glucose and sodium bicarbonate individually, as needed, he came to feel that the results obtained were distinctly superior to those obtained by the administration of Ringer's solution and dextrose alone.

The proper dosage of alkali given in this way, however, could only be ascertained with certainty by means of chemical examination of the blood, which was a distinct disadvantage in prolonged cases of diarrhea and acidosis in small infants.   In addition, to be non-irritating enough to be given subcutaneously or intraperitoneally, sodium bicarbonate solution had to be sterilized by Berkefeld filtration and brought to approximately pH 7.4 by means of the addition of carbon dioxide.   Such difficulties, it was felt, restricted considerably the use of alkali outside of hospital practice, even though its administration may have been unquestionably indicated.

With the realization that the normal kidney has a most marked

ability to select for retention or excretion those substances needed or not needed by the body, provided a sufficient amount of water is available for adequate urinary volume, we felt that a solution, to be effective against loss of either gastric or intestinal secretions, should contain: (1) water in relative abundance (i.e. should be a somewhat hypotonic solution); (2) a sufficient source of Cl' and B^+ to overcome the effects of the loss of gastric juice; (3) a sufficient excess of fixed base over fixed acid which, while being available quickly enough without kidney intervention to overcome severe acidosis, would still be released slowly enough for excretion into the urine as $BHCO_3$ in the presence of alkalosis; (4) fixed base in proper physiological ratio as regards Na, K and Ca; and (5) an antiketogenic substance.

These conditions it was thought would be fulfilled by a solution of the following composition:

$$
\begin{array}{lll}
\text{NaCl} & = 95.0 \text{ mM } B^+ \\
\text{KCl} & = 5.0 \text{ mM } B^+ \\
\text{CaCl}_2 & = 2.5 \text{ mM } B^+ \\
\text{Na lactate} & = 25.0 \text{ mM } B^+
\end{array}
$$

Such a solution has a total osmolar concentration of about 240 mM as compared with the normal plasma concentration of 320. The Cl content approximates the normal plasma concentration, the cations are in the same proportions as they occur in normal plasma and sodium lactate besides being antiketogenic provides an excess of fixed base over fixed acid when the lactate ion is removed by synthesis or oxidation. From previous clinical (7) as well as experimental (8, 9) studies, it would be expected that base would be released more or less quantitatively from combination with the lactate ion during the period of 3 or 4 hours after entrance into the blood stream, which should provide sodium bicarbonate rapidly enough in case of need but also slowly enough for excretion in case of excess.

Our experimental work on dogs was done (1) to gather more data as to the composition of normal digestive juices, (2) to study more thoroughly the effects of loss of such secretions and (3) to test the efficiency of the theoretically ideal solution mentioned above (which will hereafter be referred to as "combined" solution) in restoring the altered plasma to normal under experimentally produced conditions

of dehydration with alkalosis or acidosis. This communication will be confined to a consideration of the gastric and pancreatic secretions.

Chemical Methods

In all instances blood was obtained under oil by puncture of the femoral artery.

Chloride was determined by the method of Van Slyke (10); bicarbonate by the method of Van Slyke (11); pH by the method of Hastings and Sendroy (12); protein by the micro-kjeldahl method (13); inorganic phosphorus by the method of Briggs (14); sulphate by the method of Dennis (15); lactic acid by the method of Friedemann, Cotonio and Shaffer (16); total base by the method of Stadie and Ross (17); glucose by the method of Shaffer and Hartmann (18); NPN by the method of Folin and Wu (19); urea by the method of Van Slyke (20) and the freezing point by a thermo-couple and galvanometer as described by Darrow and Buckman (21). The osmolar concentrations were calculated as described by Darrow and Hartmann (22).

Loss of Gastric Juice

1. Chemical changes induced by loss of gastric juice through repeated lavage

Experiment No. 1.   Dog #2

In order to effect loss of gastric juice as simply as possible, and to avoid entirely operative procedures on the digestive tract which might influence quantitatively or qualitatively normal gastric secretion, dog No. 2 was permitted to eat and drink and live normally with the exception that some of the food together with the secreted gastric juice was withdrawn by gastric lavage. At least one or two feedings per day were permitted to be retained to prevent significant starvation, and water was kept at all times in the cage to provide adequate water intake during intervals between lavage. Water intake and output were not measured, but frequent observations indicated that the animal both drank and urinated fully as much as the normal animal.

The chemical changes following lavage are shown in Table I and Chart I. As would be expected, loss of the chlorine ion with increase in the bicarbonate ion (and pH) occurred in a few days time. Later, as loss of gastric juice continued, anhydremia developed, as indicated both by increase in serum protein and reduction of serum water contents. This dehydration occurred despite an apparently normal intake of water. During the third week when the Cl reduction and anhydremia were maximal, there also occurred a considerable increase
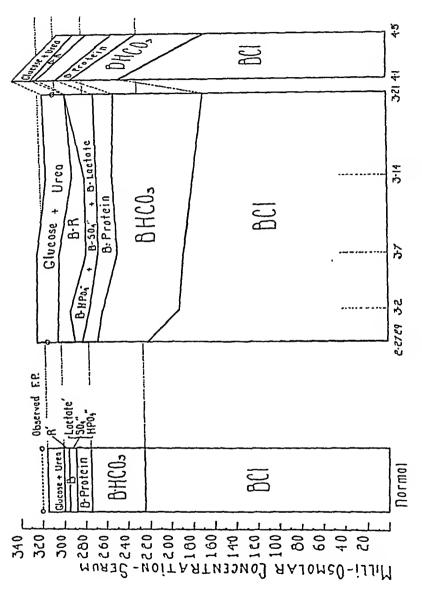
CHART I. Chemical Changes in the Blood after Gastric Lavage

TABLE I

Composition of Blood Serum of Dogs, as Affected by Loss of Gastric Juice and Therapeutic Measures

Dog No.	Date	Day of Drainage	BCl mM	BHCO₃ mM	pH	Protein gms. %	B⁺ mM	B-HPO₄ mM	B-SO₄ mM	B-Lactate mM	Total Base mM	Undetermined Acid mM	Glucose mgr. %	N.P.N. mgr. %	Urea N mgr. %	H₂O content gm. %	Total Osmolar Concentration Observed mM	Total Osmolar Concentration Calculated mM	Remarks
	Exp. 1. Loss of Gastric Juice by Lavage																		
2	2-27-29		103.8	22.1	7.40*	5.99*	12.5	1.8	2.1	2.8	151	6	100*	43.3	33.8	94.2*	312	321	Lavage started
	3- 2-29		90.0	33.6	7.50*	5.63*	12.6	3.4	2.1*	4.1	151*	5	90*	49.0	35.0*	94.5	—	319	
	3- 7-29		86.8	29.2	7.55	6.83	15.2	3.2	2.1*	1.5	150	12	90*	57.0	38.2	93.7	—	318	
	3-14-29		82.1	35.1	7.44	6.79	14.4	2.9	2.6	6.0	146	3	93	64.0	45.5	93.6		308	
	3-21-29		76.8	38.1	7.53	7.66	16.9	4.5	2.1	7.7	—		93*	67.0	39.2	92.9*	296	310	
	4- 1-29		113.0	21.3	7.35	5.83	11.8	3.1	2.1*	5.4	159	2	67	41.3	28.4	93.6	300	331	Lavage stopped
	4- 5-29		76.0	33.8	7.51	5.02	10.9	2.7	1.3	8.1	138	5	100*	46.0	26.1	92.8	274	295	" resumed
	Exp. 2. Loss of Gastric Juice by Fistula—no Significant Treatment																		
28	2-12-29	1st	109.2	18.9	7.37	6.86	14.0	2.4	1.0*	4.2	144	-5	58	36.0	25.0*	94.1	—	310	
	2-16-29	5th	56.3	32.3	7.50*	6.48	14.1	8.7	3.0*	3.4	126	11	80*	214.0	175.0	92.7	—	316	Dog died 2-17-29

Exp. 3. Loss of Gastric Juice by Fistula—Administration of Glucose and "Combined" Solution

22	4-10-29	3rd	69.0	21.7	7.40*	7.59	15.8	4.1	2.0*	9.4	131	9	80*	80*	70.0	55.5	93.4	298	282
	4-11-29	4th	56.2	31.7	7.55*	8.58	19.0	6.8	2.0*	2.4	126	8	80*	127.0	89.0*	92.4	334	281
	4-13-29	6th	99.0	21.4	7.40*	5.95	12.5	2.9	6.2	3.6	144	-2	93	27.0	17.4	94.5	303	297
1-38	5-31-29	3rd	76.7	35.0	7.55*	—	—	—	—	—	—	—	80.0	—	91.7			
	6- 1-29	4th	81.2	31.5	7.50*	6.95	15.4	—	—	1.41	—	—	38.0	—	93.6			
	6- 3-29	6th	61.0	34.9	7.55*	7.87	17.6	—	—	137	—	—	93.0	—	92.6			
8:45 a.m.	6- 4-29	7th	82.0	31.2	7.50*	5.73	12.4	—	—	3.1	136	—	—	33.0	—	93.9		
2:00 p.m.	6- 4-29		88.0	29.5	7.45*	5.28	11.3	—	—	2.8	132	—	—	30.0	—	93.4		
	6- 5-29	8th	97.2	23.0	7.40*	4.58	9.6	1.8	—	133	—	115	16.0	—	94.3			

See text for account of symptoms and therapy

* Assumed values necessary for calculating total osmolar concentration and base-binding value of protein.

in blood lactic acid and a slight but appreciable increase in NPN, and decrease in total fixed base. Also during this period the osmotic pressure as determined by the freezing point diminished very slightly. The calculated total osmolar concentrations were in good agreement with the observed.

Ten days after gastric lavage was discontinued, the blood was again studied and found normal with the exception of BCl concentration, which was above the normal.

After four days of more intensive lavage during which time altogether eighteen stomach washings were made, the Cl ion concentration dropped to a level similar to that obtained after the previous three weeks of less intensive lavage,—namely to 76 mM. Bicarbonate ion increase was in part masked by increase of lactate. The total base was diminished 21 mM and the observed osmotic pressure dropped to a distinctly subnormal level. The calculated osmotic pressure was also subnormal but to a lesser degree. The NPN increased very little during this period. A slight fall of protein concentration occurred.

2. Effects of Loss of Gastric Juice by Fistula

Experiment 2.   Dog #28

In this experiment we desired chiefly to note the rapidity and extent of plasma changes resulting from total loss of gastric juice.

On 2–12–29 the pylorus was resected under ether, the duodenal end closed and the stomach drained to the outside through a rubber tube. On 2–4–29, 3 mg. erganine were injected hypodermically to stimulate secretion of gastric juice for immediate collection and examination. 500 cc. of 5 per cent dextrose were given intraperitoneally on 2–15–29 and 2–16–29. Severe tetany and asthenia were present on the latter day. The dog was found dead on 2–17–29 the 6th day of drainage. From Table I it may be noted that by the 5th day of drainage plasma BCl concentration had fallen from 109 to 56 mM. Base bicarbonate increased only from 19 to 32 mM. This great discrepancy, 40 mM base, was well accounted for by the actual decrease in total base concentration plus the increase in phosphate and undetermined acid. NPN, 82 per cent of which was urea, increased from 36 to 214 mgs. per cent. The water content of the serum diminished without a corresponding increase in protein. The total calculated osmolar concentration changed only from 310 to 316 osm. mM. A sample of gastric juice obtained on the third day after injection of erganine showed the following composition; total Cl 133 mM, HCl 78 mM, total fixed base 27 mM, total N 507 mgs. per cent.

Experiment #3. Dog #22

In this experiment we proposed to investigate the effect on the prevention of dehydration of administration into the jejunum of: (1) dextrose solution and (2) dextrose solution and "combined" solution.

On 4-8-29 a gastric fistula was made under ether as in the previous experiment and in addition a jejunostomy was performed. Free drainage of gastric juice was noted on 4-9-29 and 200 cc. of 5 per cent dextrose were placed into the jejunum. On 4-10-29, 550 cc. were given. Tetany with convulsions developed on this day and blood was taken for chemical examination immediately after one of the latter. Changes similar to but not quite as marked as those noted in the previous experiment on the 5th day of drainage were found, with the exception that base bicarbonate increase was entirely absent, being masked by a very marked increase in lactic acid, presumably the result of convulsions. On the next day, three days after operation, BCl had diminished to 56 mM. Base bicarbonate had increased to 32 mM. Protein, phosphate, undetermined acid and NPN had increased significantly and total base had diminished to 126 mM. The total osmolar concentration observed was 334 mM, compared to a calculated osmolar concentration of 281. Signs of tetany were present on this day. In all respects, therefore, this dog appeared similar to the previous one on the day before death. On this day, however, 500 cc. of "combined" solution with 5 per cent added dextrose were given; on the next day 750 cc. were given in three divided doses, and on 4-13-29 the 5th day after operation, blood was examined chemically and found normal in all respects. At this time the dog seemed in very good shape, was able to walk and there was no signs of tetany.

Experiment No. 4. Dog #1-38

In this experiment we desired to confirm the results of the previous experiment.

On 5-29-29 a gastric fistula was made under ether by dividing the pylorus, closing the duodenal end and sewing the stomach end into the incision. Blood was examined two days later and the expected changes were found. On this day (5-31-29) 500 cc. of "combined" solution were given intraperitoneally. On the next day, the animal was more lively and the blood composition had changed in the direction of normal. No treatment was given on this day and on 6-2-29 in spite of the intraperitoneal administration of 500 cc. of 5 per cent dextrose, the dog appeared dehydrated and moribund. A relapse from the chemical standpoint was also noted (see Table I) despite the administration of dextrose solution. On 6-3-29, 500 cc. of "combined" solution were given intravenously followed by almost immediate symptomatic improvement. Later in the day 500 cc. were given intraperitoneally. On 6-4-29, 500 cc. were given intravenously at 11 a.m.

and three hours later blood was taken for study. At the same time urine was obtained. The blood showed a definite rise in BCl with a significant decrease of $BHCO_3$ and a slight fall in lactic acid. The urine was dilute. Its Cl concentration was 416 mM, $BHCO_3$ concentration 53.2 mM, NH_4 8 mM, while the total nitrogen concentration was 280 mgs. per cent. At 4 p.m. on 6–4–29 500 cc. more of "combined" solution were given intraperitoneally. This dosage was repeated on 6–5–29 at 12 noon, and at 3 p.m. on the same day blood was examined and found practically normal. The animal was killed and autopsied on 6–7–29. On that day the dog was able to walk; there were no signs of tetany. No infection was present.

It would seem therefore from the data of these experiments that the dehydration and alkalosis resulting from the loss of gastric juice is adequately relieved by administration of "combined" solution.

Loss of Pancreatic Juice

In these experiments pancreatic juice was obtained by the method previously described by one of us (23). (R. E.)

Very recently Gamble and McIver (24) published data concerning the inorganic composition of pancreatic juice as obtained by an open fistula and discussed the dehydration and acidosis which followed such loss of pancreatic juice. As might have been predicted, they observed a favorable effect on the dehydration and acidosis by the administration of sodium chloride and sodium bicarbonate. At the time of their publication we had made similar observations on two dogs concerning the nature of pancreatic juice and the effects of its complete loss by the method of intubation of the main pancreatic duct after ligation of all accessory ducts. In commenting on the two methods, Gamble and McIver concluded that the open fistula method was the superior, because they felt that premature death occurred in the intubated animals because of concomitant vomiting provoked by the intubated catheter. It would seem, however, that in reality the earlier death noted in the intubated animals (5 to 8 days as compared with 35 days) resulted from the more rapid dehydration and more marked acidosis due to the more extensive loss of pancreatic juice. In the method we used it was possible to obtain the entire 24 hour output from the entire pancreas and under sterile conditions. This is in contrast to the inevitable infection as well as the licking and consequent partial restoration of pancreatic juice in dogs with open fistulae. Infection itself leads to fibrosis of the gland and marked diminution of pancreatic secretion, an observation noted by most workers using open pancreatic fistulae. Vomiting in the intubated animals occurred only when fluid was taken into the stomach and is a manifestation of the gastric irritability which is part of the picture. Most animals seemed to realize this and took very little fluid and did not vomit. In a few dogs the desire for food and water was so great that they drank in large amounts and usually vomited afterwards.

Composition of Pancreatic Juice

Pancreatic juice as collected early after intubation, is faintly opalescent, sterile and without odor. Its total base concentration is roughly that of normal plasma, 150–160 mM and consists chiefly of sodium. Calcium averages about 4.5 mgm. per cent. Of the anions, HCO_3' is the most concentrated, averaging about 105 mM. Cl' averages about 40 mM. Protein varies from .8 to 1.9% averaging 1.2%. The albumin/globulin ratio is a little less than 1:2. Inorganic phosphorus concentration is usually less than 1 mg. per cent. Lactic acid concentration is also less than in plasma, averaging less than 1 mM. Undetermined acid averages about 5 mM. Glucose is absent and urea concentration approximates that of plasma. The total osmolar concentration both as determined and calculated also approximates that of normal plasma. The amount of pancreatic juice secreted during the first 24 hours averages between 10 and 20 cc. per hour and increases to 15–30 cc. per hour for the next few days.

The Effects of Continued Complete Loss of Pancreatic Juice

During the first 2 or 3 days of drainage, the average animal appears normal. As described in a previous paper, (23) he then develops loss of appetite, shows evidence of dehydration, but frequently not of thirst, becomes weak, drowsy, hyperpneic and dies on about the eighth day. Vomiting tends to occur particularly if food is gavaged or if the animal persists in drinking or eating, but may be entirely absent.

Plasma chemical changes are uniformly characteristic. (Table III and Chart II.) If vomiting is absent there occurs a steady fall in $BHCO_3$ until its concentration reaches about 7–8 mM when death intervenes. BCl also diminishes but usually only to a slight extent as compared with the diminution seen following loss of gastric juice. Protein concentration increases steadily and shortly before death may be extremely high, 10–13 per cent. Phosphoric, lactic and sulphuric acid concentrations increase several fold. Total base diminishes often to the extent of 25–30 mM. Undetermined acid tends to increase, but frequently remains extremely small. NPN concentration may exceed 200 mgm. per cent. Glucose in some animals is found low (in one case it was practically absent) while in others its concentration varies from the normal to 200–270 mgm. per cent.

Composition of Pancreatic Juice Obtained by Intubation

Dog No.	Date	(1) Day of Drainage	(2) 24 hr. Volume cc.	(3) HCl mM	(4) BHCO₃ mM	(5) Protein B+ gms %	(6) Protein B+ mM	(7) B-HPO₄ mM	(8) B-Lactate mM	(9) Total Base mM	(10) Undetermined Acid mM	(11) N.P.N. mg %	(12) Urea N mg %	(13) Total Osmolar Concentration Observed mM	(14) Total Osmolar Concentration Calculated mM	Remarks
							Uncomplicated Total Loss of Pancreatic Juice									
20	12-20-28	2nd	550	54.7	84.5	1.9	4.7	0.0	0.1	154	10	34.0	24.0*	319	307	Juice sterile
	12-24-28	6th	365	76.7	66.1	1.2	3.0	0.3	0.7	149	2	44.0	31.0*	313	311	"
	12-26-28	8th	320	73.3	61.1	1.4	3.6	0.2	0.2	141	3	83.6	58.5*	304	309	"
	12-27-28	9th	145	62.2	60.7	1.1	2.7	0.0	0.4	130	4	220.0	154.0*	332	331	Day of death
							Effects of Ringer's Solution Administration									
23	12-29-28	2nd	270	47.0	84.5	1.2	—	—	—	139	—	32.0	—	—	—	Juice sterile
	12-30	3rd	390	36.1	103.5	1.3	—	—	—	147	—	18.4	—	—	—	"
	12-31	4th	390	42.6	83.7	1.2	—	—	—	144	—	12.0	—	—	—	500 cc. Ringer's Sol.
	1-1-29	5th	365	57.2	73.2	1.2	—	—	—	141	—	19.0	—	—	—	" "
	1-2	6th	440	61.1	77.1	1.5	—	—	—	148	—	18.0	—	—	—	" "
	1-3	7th	600	53.0	85.8	1.4	—	—	—	158	—	24.0	—	—	—	500 cc. Ringer's Sol.
	1-4	8th	720	51.4	84.8	1.2	—	—	—	158	—	14.4	—	—	—	500 " "
	1-5	9th	610	56.2	80.0	1.3	—	—	—	143	—	25.6	—	—	—	500 " "
	1-6	10th	390	46.0	79.0	2.1	—	—	—	150	—	29.3	—	—	—	500 " "
	1-7	11th	370	36.1	82.0	1.6	—	—	—	139	—	24.0	—	—	—	500 " "
	1-8	12th	320	36.9	79.0	1.5	—	—	—	135	—	18.0	—	—	—	500 " "
	1-9	13th	325	30.9	86.0	1.6	—	—	—	140	—	23.2	—	—	—	Juice infected 500 cc. Ringer's Sol.
	1-10	14th	200	36.9	83.0	1.9	—	—	—	133	—	36.0	—	—	—	" 500 "
	1-11	15th	200	42.1	83.0	1.8	—	—	—	140	—	26.0	—	—	—	" 500 "

* Assumed values necessary for calculating total osmolar concentration.

Effects of "Combined" Solution Administration

27	1-29-29	2nd	300	38.9	101.2	1.9	—	—	—	163	57.6	—	—	—	Juice sterile
	1-30	3rd	550	37.9	103.7	1.4	—	—	—	153	38.0	—	—	—	"
	1-31	4th	560	43.4	98.2	1.9	—	—	—	147	44.0	—	—	—	" infected 800 cc "combined" Sol.
	2-1	5th	390	39.0	91.4	2.4	—	—	—	141	50.0	—	—	—	" " 400 " "
	2-2	6th	560	30.9	95.5	1.4	—	—	—	137	44.0	—	—	—	" " 400 " "
	2-3	7th	115	37.9	85.7	1.4	—	—	—	142	68.0	—	—	—	" " 400 " "
	2-4	8th	275	37.9	78.6	1.4	—	—	—	132	92.0	—	—	—	" " 800 " "
	2-5	9th	305	35.1	83.1	1.6	—	—	—	131	80.0	—	—	—	" " 800 " "
	2-6	10th	160	12.2	71.8	1.6	—	—	—	128	80.0	—	—	—	" " 800 " "
	2-7	11th	100	56.8	42.0	?	—	—	—	135	—	—	—	—	" " 800 " "
	2-8	12th	160	73.5	45.7	?	—	—	—	149	427?	—	—	—	" " Dog killed
1	2-27-29	2nd	260	39.0	107.0	1.0	—	—	—	160	32.0	—	—	—	Juice sterile
	2-28	3rd	135	45.0	102.2	1.1	—	—	—	158	23.0	—	—	—	"
	3-1	4th	130	61.0	79.0	1.1	—	—	—	147	18.4	—	—	—	Juice infected
	3-2	5th	400	65.0	63.7	1.3	—	—	—	142	51.3	—	—	·	" " 1000 cc. "combined" Solution on 3-2
															" " 1000 " " " 3-3
															500 " " " 3-4
	3-4	7th	200	66.0	41.7	1.4	—	—	—	135	260.0	—	—	—	1000 " " " 3-5
															750 " " 3-6

The water content of the serum diminishes usually from 94 to 91 gms. per 100 cc. The plasma volume as compared with the cell volume decreases enormously, however, sometimes being only one-fifth the total blood volume. The total osmolar concentration both as observed and calculated changes but little.

If marked vomiting occurs, $BHCO_3$ loss may be partly or entirely masked, while BCl diminution is intensified.

Early infection of the pancreatic juice and of the pancreas may also alter the course of events somewhat, as mentioned earlier. Usually infection results in diminution of pancreatic secretion, and delays fatal changes somewhat (see Table II). In Dog ⚡25 glucose was practically absent from the blood on the 13th day (day of death) and an extremely high concentration of lactic acid was present. Apparently either glycolysis occurred in vivo or else developed extremely rapidly after removal of the blood.

Renal activity (Chart II) is such as to compensate for the plasma changes. The urine diminishes in volume, contains almost no bound HCO_3 and therefore becomes highly acid, becomes free of Cl and concentrated as regards HPO_4, ammonia and urea. Fixed base is much diminished. A heavy trace of albumin and numerous granular casts are usually present.

The pancreatic juice changes somewhat in composition as dehydration and acidosis increase. (Table II, Chart II.) It diminishes in volume and becomes considerably less rich in base bicarbonate, but remains very alkaline as compared to the plasma. BCl increases somewhat in concentration. Its total fixed base concentration steadily diminishes but usually not as rapidly or to the extent that plasma base diminishes. NPN and urea continue to approximate their plasma concentrations, probably because of simple filtration.

It would seem therefore that secretion of pancreatic juice, as in the case of gastric juice secretion, continues more or less unaltered despite the fact that its loss leads to the rapid death of the animal. Renal activity while very important in compensating for loss of water, fixed base and Cl, is insufficient to prevent death in the absence of sufficient mineral intake.
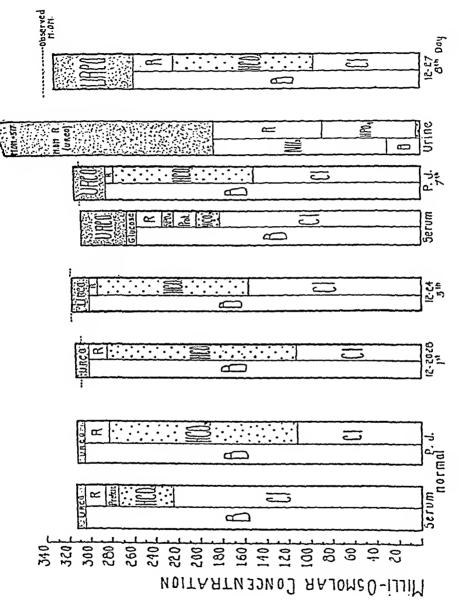
CHART II. Chemical Changes in the Blood, Pancreatic Juice, and Urine Associated with Continued Total Loss of Pancreatic Juice

TABLE III

Composition of Blood Serum as Affected by Complete Loss of Pancreatic Juice, Complicating Factors Such as Infection, Vomiting, and Incomplete Loss, and by Therapeutic Measures

Dog No.	Date	Day of Drainage	BCl mM	BHCO₃ mM	pH	Protein gms. %	Protein +B mM	B-HPO₄ mM	B-SO₄ mM	B-Lactate mM	Total Base mM	Undetermined Acid mM	Glucose mgs. %	N.P.N. mgs. %	Urea N mgs. %	H₂O content gms. %	Total Osmolar Obs. mM	Total Osmolar Calc. mM	Remarks
Uncomplicated Complete Loss of Pancreatic Juice
16	11-26-28	7th	98.8	6.2	7.17	8.49	15.8	4.1	2.0*	5.7	135	4	135	70.0	49.0*	92.2*	—	300	
20	12-26-28	8th	80.7	9.6	7.33	9.34	18.8	5.8	3.0*	3.0	117	−4	155	147.0	103.0*	90.4	—	285	
Complete Obstruction of Intubation Tube—Marked Vomiting—No Loss of Pancreatic Juice
19	12-15-28	4th	81.0	31.7	7.51	7.22	15.8	3.8	2.0*	4.8	173?	34?	148	68.2	47.8	93.2*	—	367?	
Early Infection of Pancreatic Juice
11	4- 6-29	14th	87.0	7.2	—	—	—	—	—	—	107	—	—	100.0	—	—	—	—	
25	4- 6-29	13th	97.8	14.0	7.30	5.50	10.8	3.9	1.9	16.5	158	13	20.0	70.0	49.0*	94.5	315	335	
3-10	4- 9-29	7th	80.0	7.7	7.15	10.8	19.9	5.5	8.6	6.6	122	−6	268.0	233.0	92.0	91.9	320	296	
7	5-21-29	5th	83.0	13.1	7.31	7.78	13.6	5.4	2.0*	1.5	133	14	80.0*	96.6	67.7*	91.6	—	306	
Effects of Administration of Ringer's Solution
23	12-31-28	4th	90.6	12.0	7.33	8.23	16.5	3.3	1.0*	5.0	140	12	146	31.6	16.0*	92.7	—	294	Therapy begun
23	1- 5-29	9th	90.0	8.8	7.31	7.46	14.8	3.7	1.0*	3.0	129	8	127	46.7	24.0*	91.9	—	277	
23	1-11-29	15th	95.8	14.2	7.32	6.56	13.1	2.7	1.0*	2.0	134	5	148	30.0	15.0*	95.0	—	278	

Effects of Administration of "Combined" Solution

27	1-28-29	1st	97.3	20.1	7.44	—	8.98	17.8	3.0*	—	1.0*	4.2	123	—	191	38.4	—	44.0*	91.5	—	—	269	Therapy begun
	1-31-29	4th	86.4	9.4	7.30*	—	5.45	10.9	1.4	—	1.0*	2.8	144	3	100*	63.0	13.5*	93.5	—	—	302	
	2- 8-29	12th	95.0	21.9	7.33	—	6.39	13.0	3.1	—	2.6	2.2	147	11	97	27.0	15.3	93.9*	315	304		
1	2-27-29	2nd	103.2	19.6	7.35*	—	8.12	16.1	4.9	—	3.0*	1.5	144	4	100*	30.0	69.0	92.5*	—	311	Therapy begun	
	3- 2-29	5th	76.2	14.8	7.30*	—	5.00	10.9	2.5	—	1.0*	2.5	141	24	80*	123.6	11.4	95.0*	—	304		
	3- 7-29	10th	95.2	28.0	7.51								1.5	148	9	100*	25.0						

* Assumed values necessary for calculating total osmolar concentration and base-binding value of protein.

*The Effects of the Administration of Dextrose, Ringer's and "Combined"
Solutions*

When only water and dextrose solution are administered, no note-worthy diminution of dehydration and acidosis or prolongation of life seems to occur.

Administration of Ringer's solution, however, (Table III) in amounts somewhat greater than the volume of the pancreatic juice lost has a decided beneficial effect. After administration of Ringer's solution to Dog #23 (Table II) was begun, pancreatic juice, which had diminished in volume and $BHCO_3$ concentration, again increased in volume and alkalinity. The dog showed symptomatic improvement. He became lively and his appetite returned. Urinary output increased and renal activity, largely through excretion of chloride bound to ammonia with resultant retention of fixed base, was sufficient to prevent plasma $BHCO_3$ from falling to a fatal level. It is to be noted, however, that $BHCO_3$ continued to diminish for a number of days after beginning the administration of Ringer's solution and then increased but did not nearly reach the normal level by the 15th day.

With the administration of "combined" solution almost immediate relief of dehydration and acidosis was accomplished (Table III). Kidney intervention, while necessary for the excretion of such accumulated acids as phosphoric and sulfuric and of excess non-protein nitrogen, was unnecessary for a rapid restoration to normal of plasma $BHCO_3$ and pH.

It would seem, therefore, that the "combined" solution is effective in relieving dehydration due to loss of fixed base and fixed acid, regardless of whether "acidosis" or "alkalosis" exists. Severe renal insufficiency should not prevent plasma $BHCO_3$ restoration. Theoretically, however, it might delay excretion of $BHCO_3$ excess.

An experimental study of this problem will be reserved for a later paper.*

SUMMARY AND CONCLUSIONS

The composition of gastric and pancreatic juices and the effects of their loss on the composition of the body fluids were studied. Loss

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* A preliminary report on the clinical use of the "combined" solution was given by one of us (A. F. H.) at the Forty-first Annual Meeting of the American Pediatric Society.
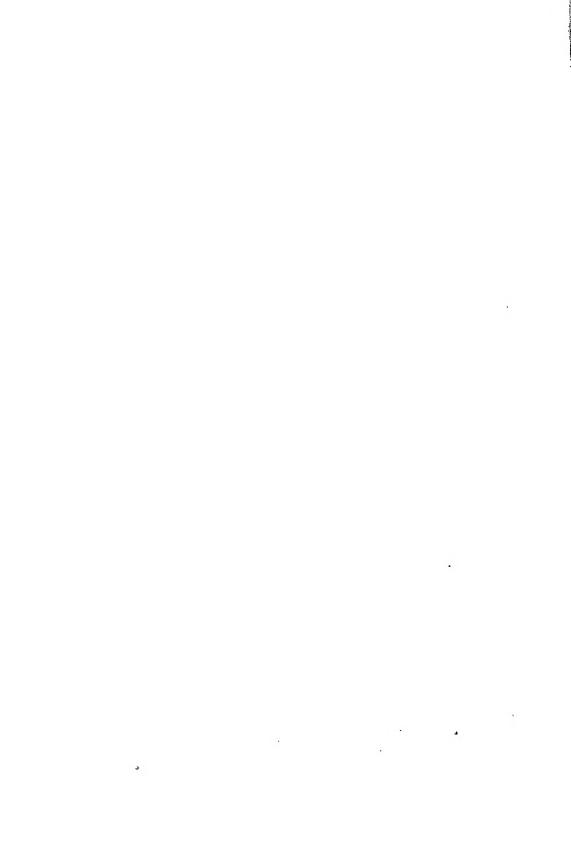
of gastric juice by removing water and chloride ions only partly neutralized by fixed base results in dehydration and alkalosis.

Loss of pancreatic juice by removing water and a relative excess of fixed base results in dehydration and acidosis.

Normal conditions in the body may be restored after the loss of either gastric or pancreatic juice by the administration of a combined solution, which provides (1) water in abundance because of its hypotonicity (2) an adequate source of the fixed anion Cl' and of the cations Na+, K+, and Ca++ in proper physiological ratio and (3) an excess of fixed base over fixed acid in the form of B-lactate.

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SEROLOGICAL DIFFERENTIATION OF STERIC ISOMERS (ANTIGENS CONTAINING TARTARIC ACIDS)

SECOND PAPER
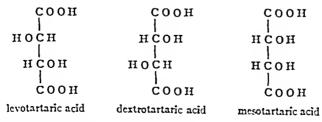
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In experiments reported already (1) it has been demonstrated that the presence of optically isomeric groups in two otherwise identical antigens suffices to bring about a difference in their serological properties. The case examined was that of the levo- and dextro-phenyl (paraaminobenzoylamino) acetic acids which after diazotization were combined with proteins. The resulting antigens were distinctly different when tested with the corresponding precipitating immune sera. Considering that steric isomerism presumably is of significance for the' specificity of natural antigens, it seemed desirable to extend the studies to other instances and also to proceed to the examination of compounds containing more than one asymmetric carbon atom.

For this purpose the tartaric acids were chosen which, as is well known, contain two asymmetric carbon atoms and which exist in three isomeric forms, namely, levo-, dextro-, and mesotartaric acid, aside from the racemic mixture of *l*- and *d*-acid. The following formulae represent the three forms

COOH	COOH	COOH
HOCH	HCOH	HCOH
HCOH	HOCH	HCOH
COOH	COOH	COOH
levotartaric acid	dextrotartaric acid	mesotartaric acid

A method for attaching these substances to protein was found in

preparing first a compound with paraphenylenediamine in which one of the amino groups is linked to one of the carboxyls of the tartaric acid according to the following formula: $NH_2C_6H_4NHCO(CHOH)_2COOH$. This substance was diazotized and coupled to protein. After a few unsuccessful attempts to prepare this acyl derivative directly by condensation of tartaric acid with paraphenylenediamine, it was found possible to combine tartaric acid with paranitraniline and to reduce the resulting substance to the amino compound.

EXPERIMENTAL

Levo- and mesotartaric acids were prepared according to the methods of Holleman (2) and Marckwald (3). The meso acid was obtained in the form of its calcium salt which forms characteristic square crystals easily distinguishable from those of the racemic mixture. From this calcium salt the free acid was isolated by adding less than the calculated amount of a dilute solution of sulfuric acid and allowing the material to stand at room temperature for 1 day. The filtered solution was concentrated to a small volume at 40° by vacuum distillation. The mesotartaric acid crystallized on cooling and was dried *in vacuo* at 45° (M.Pt. after two recrystallizations 142–143°C.)

Frankland and Slator (4) prepared a ditoluidide of tartaric acid $(C_7H_7NHCO)_2$ $(CHOH)_2$ by heating p-toluidine (2 mols) and tartaric acid (1 mol) for 10 hours in an oil bath at 180–185°. They established by subsequent hydrolysis of the substance that the tartaric acid had not undergone any appreciable racemization during the process.

Applying a similar method to paranitraniline and using instead of 2 mols only 1 mol of paranitraniline for 1 mol of tartaric acid we found that besides a non-acid product (probably the dinitranilide of tartaric acid) paranitrotartranilic acid $(NO_2C_6H_4NHCO(CHOH)_2COOH)$ in good yield was also obtained. Accordingly the derivatives of levo-, dextro-, and mesotartaric acid were prepared by the following process:

15 gm. of tartaric acid were ground with 13.8 gm. of paranitraniline and heated (in a test tube) in a paraffin bath at 170°C. When, after about 5 minutes, bubbles began to form in the molten mass the temperature was lowered to 155–160°. In the case of the levo- and dextro-tartaric acid but not in that of the meso-acid the fluid began to thicken after about 15 minutes and later solidified. After heating for about 40 minutes in all, the mass was broken up by heating with water and a little alcohol, the volume was brought to 600 cc. with water and a solution of NaOH was added until the liquid reacted neutral to litmus. The insoluble material was removed by filtration and the solution was concentrated on the steam bath to a volume of 200 cc. A little insoluble material which separated was filtered off after cooling to room temperature and enough 10 per cent hydrochloric acid was added to make the solution acid to congo red. The paranitrotartranilic acid pre-

cipitated as a light yellow powder which was filtered off, washed with water and dried *in vacuo*. The yield was approximately 12 gm. of the levo- and dextro-compounds and somewhat less of the meso-compound. Recrystallized from water the levo- and dextro acids form fine yellow needles. The meso acid crystallizes in pale yellow microscopic platelets.

Levo-paranitrotartranilic acid $C_{10}H_{10}O_7N_2$.   M.Pt. 211–212°.

Analysis: calculated: C 44.44 H 3.70

found: " 44.76 " 3.40

270 mg. neutralized 9.95 cc. 1/10 N NaOH; calculated: 10 cc. A 1% solution in methyl alcohol gave at 22° a rotation of −2.40 in a 2 dm. tube with sodium light, $[\alpha]_D$ −120.

Dextro-paranitrotartranilic acid M.Pt. 212–213°.

270 mg. neutralized 9.93 cc. 1/10 N NaOH; calculated: 10 cc. A 1% solution in methyl alcohol gave at 22° a rotation of +2.40 in a 2 dm. tube, $[\alpha]_D$ +120.

Meso-paranitrotartranilic acid M.Pt. 193–194°.

270 mg. neutralized 9.95 cc. 1/10 N NaOH; calculated 10 cc. A 1% solution in methyl alcohol showed no rotation.

Reduction of Nitrotartranilic Acid to the Amino Compound.—20 gm. of crude finely ground para-nitrotartranilic acid were suspended in 1.5 liters of ½ normal hydrochloric acid. About 36 gm. of zinc dust were added in small portions over a period of 15 minutes with constant stirring. The paranitrotartranilic acid disappeared gradually within ½ hour. The excess of zinc was filtered off and to the solution which showed faint acidity to congo red, 70 gm. of sodium acetate were added and hydrogen sulfide was passed through until the zinc had been completely precipitated. After filtration the solution was neutralized with sodium hydroxide. In the case of the meso-paraaminotartranilic acid it was neutralized before filtering off the zinc sulfide to avoid a loss of substance by precipitation in the acid solution. The solution was concentrated at 40° by vacuum distillation to a volume of 200 cc. and after removing a trace of insoluble material it was cooled in a freezing mixture and enough concentrated hydrochloric acid was added to make it weakly acid to congo. The paraaminotartranilic acid came out of solution and was filtered off. It was washed with water, alcohol and ether and dried *in vacuo*. The yield was approximately 10 gm. By recrystallization of the levo- and dextro-compounds from 25 parts of water with the addition of some decolorizing carbon they were obtained in the form of microscopic white needles or platelets.

The meso-compound which was almost white was purified by dissolving in water and the required amount of sodium hydroxide, and reprecipitation with acid; white microscopic rectangular platelets after recrystallization from water.

Analyses:

Levo-paraaminotartranilic acid $C_{10}H_{12}O_5N_2$.

240 mg. neutralized 9.85 cc. 1/10 N NaOH; calculated 10 cc.

Kjeldahl nitrogen analysis: Found N. 11.76%; calculated 11.67%.

A water solution containing 480 mg. of the substance and 2.6 cc. of normal HCl in a volume of 15 cc. gave at 25°C. a rotation of −6.31 in a 2 dm. tube; $[\alpha]_D$ −98.6.

Dextro-paraaminotartranilic acid $C_{10}H_{12}O_5N_2$
240 mg. neutralized 9.90 cc. 1/10 N NaOH; calculated 10 cc.
Kjehldahl nitrogen analysis: Found N. 11.55%, calculated 11.67%.
A water solution containing 480 mg. of the substance and 2.6 cc. of normal HCl in a volume of 15 cc. gave at 25°C. a rotation of +6.35 in a 2 dm. tube; $[\alpha]_D$ +99.2.

Meso-paraaminotartranilic acid $C_{10}H_{12}O_5N_2$
240 mg. neutralized 9.90 cc. 1/10 N NaOH; calculated 10 cc.
Kjehldahl nitrogen analysis: Found N. 11.62%; calculated 11.67%.
The amino compounds became dark but did not melt when heated to 285°C.

The meso-paraaminotartranilic acid obtained must consist of a racemic mixture of two optically active compounds since the combination with paranitraniline destroys the symmetry of the molecule. This circumstance does not interfere at all with the conclusions to be drawn from the following experiments and consequently no attempt was made to resolve the product into its components.

The levo-, dextro-, and meso-paraaminotartranilic acids will be designated as *l*-, *d*-, and *m*-acid and likewise the azoproteins prepared from these amino acids and the immune sera obtained by immunization with the azoproteins will be referred to as *l*-, *d*-, and *m*-antigens, and *l*-, *d*-, and *m*-immune sera, respectively.

Preparation of the l-, d-, and m-Antigens for Immunization.—The *l*- and *d*-paraaminotartranilic acids were coupled to protein in the following manner:

7.6 gm. were dissolved in 200 cc. water and 85 cc. of normal HCl and diazotized with the required amount of sodium nitrite at a temperature of 0–5°C. with starch iodide paper as indicator. The diazo solution was diluted with ice water to a volume of 1200 cc.

800 cc. of this solution were added to a cold mixture of 500 cc. horse serum and 100 cc. normal sodium carbonate and the mixture, chilled with ice, was kept weakly alkaline to phenolphthalein by adding frequently small quantities of sodium carbonate solution. The coupling proceeded slowly (test with alkaline R salt solution for the presence of free diazo compound) and the diazo compound was used up after about 1½ hours. Then the remaining 400 cc. of diazo solution and 50 cc. of normal sodium carbonate were added (further addition of sodium carbonate and cooling as above). The coupling was finished after about 1 hour. Under the conditions described the meso compound coupled faster but for the sake of uniformity the operations were carried out in exactly the same manner as with the *l*- and *d*- substances.

By acidification with hydrochloric acid the azoprotein was precipitated and after filtration it was dissolved in a small volume of water by addition of a little normal sodium carbonate. It was reprecipitated from this solution with a large quantity of alcohol. The precipitated azoprotein was ground in a mortar to a thin paste, brought up to a volume of 950 cc. with water and the necessary amount of a salt solution to make the salt concentration approximately 1%. As a preservative 50 cc. of 5% phenol solution were added.

Immunization.—Three batches of six rabbits each were injected intraperitoneally at weekly intervals. Each rabbit received 12 cc. of the antigen per injection. Test bleedings were made 1 week after the third and the fourth injections. Three to four sera of sufficient strength were obtained in each lot after three or four injections.

Antigens for the Tests.—These were prepared in the same way as the antigens for immunization, chicken serum being used instead of horse serum. The azoproteins were precipitated with acid, washed with water and brought into solution by means of sodium carbonate. The quantity of antigen in the solution was determined by precipitation with alcohol and weighing the dried substance. The dilutions given in the tables are in terms of a 5% stock solution.

The intensity of the reaction is indicated as follows: o, f. tr. (faint trace), tr. (trace), $\pm$, $+$, $+\pm$, etc.

The experiments presented in Tables I to III show an almost complete specificity of the *l*- and *d*- antigens. The *l*- and *d*- immune sera give rather weak group reactions with the *m*-antigen; the *m*-immune sera gave practically no group reactions.

Tests were also made with an antigen made from racemic tartaric acid. It was found that this substance, as was to be expected, reacts like a mixture of *l*- and *d*-antigens, *i.e.*, it is precipitated by both the *l*- and *d*-immune sera like the homologous antigens only somewhat weaker according to the lower concentration of the respective homologous antigens. With the *m*-immune sera there were only faint reactions similar to those of the *l*- and *d*-antigens. These results with the racemic preparation are not tabulated.

Other immune sera, namely, two *l*-, three *d*- and three *m*- sera gave results entirely in agreement with those presented in the tables.

In the following experiments presented in Tables IV,*a*, IV,*b*, and IV,*c*, the inhibiting effect on the precipitin reaction of the tartaric acids, the paraaminotartranilic acids and some other substances used for

TABLE I, a

To 0.2 cc. of the diluted antigens (prepared with chicken serum) were added 2 capillary drops of l-immune serum.

Readings taken after:	l-Antigen Dilution 1:				d-Antigen Dilution 1:				m-Antigen Dilution 1:			
	20	100	500	2500	20	100	500	2500	20	100	500	2500
1 hr. at room temperature	±	+	+	tr.	0	0	0	0	0	tr.	0	0
3 hrs. at room temperature	+	+±	+±	tr.	0	0	0	0	f. tr.	tr.	f. tr.	0
Night in ice box	+±	++	++	+	0	0	f. tr.	0	±	±	tr.	0

TABLE I, b

To 0.2 cc. of the diluted antigens were added 4 capillary drops of l-immune serum.

Readings taken after:	l-Antigen Dilution 1:				d-Antigen Dilution 1:				m-Antigen Dilution 1:			
	20	100	500	2500	20	100	500	2500	20	100	500	2500
1 hr. at room temperature	+	+±	+	tr.	0	0	0	0	f. tr.	tr.	f. tr.	0
3 hrs. at room temperature	+	++	+±	±	0	0	0	0	tr.	tr.	tr.	0
Night in ice box	++±	+++	++±	+	0	f. tr.	0	0	±	+	tr.	0

TABLE II, a

To 0.2 cc. of the diluted antigens were added 2 capillary drops of d-immune serum.

Readings taken after:	l-Antigen Dilution 1:				d-Antigen Dilution 1:				m-Antigen Dilution 1:			
	20	100	500	2500	20	100	500	2500	20	100	500	2500
1 hr. at room temperature	0	0	0	0	tr.	+	+	tr.	0	tr.	f. tr.	0
3 hrs. at room temperature	0	0	0	0	tr.	+	+	±	f. tr.	tr.	f. tr.	0
Night in ice box	0	0	f. tr.	0	±	++	++	+±	±	±	tr.	0

TABLE II, b

To 0.2 cc. of the diluted antigens were added 4 capillary drops of d-immune serum.

Readings taken after:	l-Antigen Dilution 1:				d-Antigen Dilution 1:				m-Antigen Dilution 1:			
	20	100	500	2500	20	100	500	2500	20	100	500	2500
1 hr. at room temperature	0	0	0	0	+	+±	+	tr.	tr.	tr.	f. tr.	0
3 hrs. at room temperature	0	0	0	0	+	+±	+	tr.	tr.	tr.	tr.	0
Night in ice box	0	0	0	0	++±	+++	++±	+	+	+	tr.	0

TABLE III, a

To 0.2 cc. of the diluted antigens were added 2 capillary drops of m-immune serum.

Readings taken after:	l-Antigen Dilution 1:				d-Antigen Dilution 1:				m-Antigen Dilution 1:			
	20	100	500	2500	20	100	500	2500	20	100	500	2500
1 hr. at room temperature	0	0	0	0	0	0	0	0	±	+	+	±
3 hrs. at room temperature	0	0	0	0	0	0	0	0	±	+	+±	±
Night in ice box	0	0	f. tr.	0	0	0	0	0	++	++±	++±	+±

TABLE III, b

To 0.2 cc. of the diluted antigens were added 4 capillary drops of m-immune serum.

Readings taken after:	l-Antigen Dilution 1:				d-Antigen Dilution 1:				m-Antigen Dilution 1:			
	20	100	500	2500	20	100	500	2500	20	100	500	2500
1 hr. at room temperature	0	0	0	0	0	0	0	0	±	+	+	tr.
3 hrs. at room temperature	0	0	0	0	0	0	0	0	±	++	+±	±
Night in ice box	0	f. tr.	f. tr.	0	0	0	0	0	++±	+++	+++	+

TABLE IV, *a*

l-Antigen and l-Immune Serum

Readings taken after:	1*	2	3	4	5	6	7	8	9	10	11	12	Control
15 mins. at room temperature	f. tr.	±	tr.	0	±	f. tr.	+	±	±	+	+	+	+
3 hrs. at room temperature	±	+	±	0	+	±	+±	+	+	+±	+±	+±	+±
Night in ice box	+	++	+	0	+	±	++	+±	+±	++	++	++	++

TABLE IV, *b*

d-Antigen and d-Immune Serum

Readings taken after:	1	2	3	4	5	6	7	8	9	10	11	12	Control
15 mins. at room temperature	±	0	tr.	tr.	0	f. tr.	+	±	tr.	±	+	+	+
3 hrs. at room temperature	+	±	+	±	0	tr.	+±	+	±	+±	+±	+±	+±
Night in ice box	+±	+	++	++	0	±	++	++	+±	+±	+±	+±	+±

TABLE IV, *c*

m-Antigen and m-Immune Serum

Readings taken after:	1	2	3	4	5	6	7	8	9	10	11	12	Control
15 mins. at room temperature	+	+	tr.	tr.	±	0	+	±	±	±	±	+	+
3 hrs. at room temperature	+±	+±	±	+	+	0	+±	+±	+±	+±	++	+±	++
Night in ice box	++±	++±	+±	++±	++	0	+++	++	++	+++	++±	+++	++±

*The numbers 1 to 12 refer to the substances tested for inhibition.

comparison was tested. The compounds are enumerated as follows: (1) levo-tartaric acid, (2) dextro-tartaric acid, (3) meso-tartaric acid, (4) levo-paraaminotartranilic acid, (5) dextro-paraaminotartranilic acid, (6) meso-paraaminotartranilic acid, (7) succinic acid, (8) l-malic acid, (9) d-l-malic acid, (10) lactic acid, (11) benzoic acid, (12) acetic acid.

0.2 cc. of the antigen (diluted 1:500) were mixed with 0.05 cc. of a neutral solution containing 1 millimol in 10 cc. of the substances indicated. To this 4 capillary drops of the homologous immune serum were added. The control tube contains only antigen and immune serum.[1]

TABLE V

The inhibiting substances used were: (4) l-paraaminotartranilic acid, (5) d-paraaminotartranilic acid, (6) m-paraaminotartranilic acid, (13) l-paranitrotartranilic acid, (14) d-paranitrotartranilic acid.

	Readings taken after	4	5	6	13	14	Control
l-antigen and l-immune serum	15 mins. at room temperature	0	±	tr.	0	±	+
	3 hrs. at room temperature	0	+	±	0	+	+±
	Night in ice box	0	+±	+±	0	+±	++
d-antigen and d-immune serum	15 mins. at room temperature	±	0	tr.	tr.	0	+
	3 hrs. at room temperature	+	0	±	±	0	+±
	Night in ice box	+±	0	+±	+±	0	+±
m-antigen and m-immune serum	15 mins. at room temperature	±	±	0	tr.	±	±
	3 hrs. at room temperature	+	+	0	+	+	+±
	Night in ice box	+±	+±	0	+±	+±	++

Table V represents inhibition tests with l-, d- and m-paraaminotartranilic acid and l- and d-paranitrotartranilic acid where 0.05 cc. of a neutral solution containing in 10 cc. 0.25 millimol of these substances was added to the antigen. The m-paranitrotartranilic acid could not be used since a precipitation occurred on mixing with saline solution.

DISCUSSION AND SUMMARY

The experiments reported confirm the results of our previous studies and demonstrate again the striking influence of the steric constitution

[1] In the details given for the tests in a previous paper (1), Table IV c, it should read "0.5 millimol in 10 cc."

on serological properties. It would seem that in this respect the specificity of serum reactions is analogous to that of ferments. However, while in the investigations with ferments one is limited to those found in nature, by our method antibodies can be produced at will, which act on chosen substances.

In particular one sees that the change in the spatial configuration with regard to one asymmetric carbon atom is sufficient to cause a pronounced serological difference, as appears from a comparison of the reactions of either the *l*- or *d*-antigen with those of the *m*-antigen. Since the change of the levo- into the dextro-acid would involve a rearrangement of the groups around both asymmetric carbon atoms one may suppose that there is a greater serological difference between these two acids than between either and the *m*-tartaric acid. This reasoning is supported by the fact that the *l*-immune sera showed almost no reactions on the *d*-antigen and vice versa while there were weak group reactions of the *l*- and *d*-immune sera with the *m*-antigen. A question which requires further investigation is whether the marked serological distinction between steric isomers depends on the nature of the radicals connected with the asymmetric carbon atom, *i.e.*, whether any radical will produce an effect of the same order as polar groups like COOH or OH (*c.f.* Reiner (5)).

The fact that the specificity of the reactions is entirely determined by the substances linked to the protein and not by the protein molecule itself is in the first place due to the method of testing, namely, the use of a protein different from that employed for immunization. Besides one has to consider that, owing to the considerable number of tyrosine and histidine groups with which the azocompounds can combine, one may visualize the protein molecules, studded, as it were, with the groups artificially introduced.

The application of the findings presented, to the problem of specificity in natural antigens, especially those of carbohydrate nature, is suggested because the chemical constitution of tartaric acid is closely allied to that of the sugar acids which, from the work of Avery, Heidelberger and Goebel are known to form essential parts of the bacterial carbohydrate haptens. Since these haptens are high molecular compounds consisting of structurally different combinations of sugars and various carbohydrate acids it is easily conceivable

that there exists an almost unlimited number of such specific substances.

A special point is brought out by the inhibition tests. These tests distinguish definitely between the uncombined *l-* and *d-*tartaric acids themselves, while in contrast to the direct precipitin reactions there is no definite distinction between *l-* or *d-* and the *m-*tartaric acid, in the tests with *l-* or *d-*immune serum. The *m-*immune serum however differentiates clearly the *m-*tartaric acid from the two other acids. In comparison with the simple tartaric acids a considerably greater inhibiting effect and a more marked specificity similar to that of the precipitin tests is exhibited by the amino- or nitrotartranilic acids. This indicates that the antibodies involved do not act upon the tartaric acid part of the molecule only but attach themselves also to the aromatic nucleus and hence may be supposed to possess several distinct binding groups. This view suggests a further study of substances of complicated chemical structure.

In the present connection it is pertinent to state that in general the conclusions drawn from the inhibition tests are based upon reactions with substances whose chemical constitution is known in its entirety, and that of course proteins play no part whatsoever in the specificity of these phenomena.

SUMMARY

In continuation of studies on the stereochemical specificity of serum reactions, antigens were examined containing the acyl radicals of the levo-, dextro-, and meso-tartaric acids. It was found that in this case also, immune sera can readily be obtained which differentiate sharply the three antigens identical in every other respect but possessing stereoisomeric groups.

Since the tartaric acids by their chemical constitution belong to the same class of substances as sugar acids the results have a bearing upon the question of the specificity of natural antigens containing carbohydrates such as have been described by Avery and Heidelberger.

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THE ANTIBODY-FORMATION BY POLYSACCHARIDS

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That antibodies are formed for lipoids had long been doubted. Recently, however, this doubt has been cleared away by the production of the antibodies (1) (2) in animals injected with lipoids and serum proteins.

It has occurred to me that polysaccharids, which belong to the same sort of emulsoids, might be endowed with antigenic properties and that their action as antigens might be facilitated by using serum proteins or other substances as vehicle.

The fact that glycolytic ferment develops in the animal body after the injection of sugar has been demonstrated by Weinland, Abderhalden, and others. There is, however, little literature dealing with the antigenic nature of sugar. Heidelberger and Avery (3) (4) have isolated a substance which bore close resemblance to polysaccharids from the pneumococci. This substance precipitated antipneumococcic serum, but immunization experiments with it have given a negative result. Rokuro Kondo (5) obtained an antilipoidal serum by the use of a mixture of lipoids with inulin.

I have experimented with inulin, soluble starch and dextrine, and succeeded in obtaining antisera against all of them. The results were reported in the December issue of the *Japan Medical World*, Home Edition in 1927 (6), and in the *Saikingaku Zasshi* (*Journal of Bacteriology*) April, 1928 (7). I shall deal with the same matter in this report, reviewing all I have reported previously in addition to what I have done since.

Methods

The three kinds of polysaccharids used in my experiments were of Merck make. I prepared solutions of them and injected them into the postauricular veins of rabbits daily for some time. On the 5th to 6th day from the last injection the blood of the animals was collected, and the serum was separated and inactivated

at 56°C. for ½ hour. The test was made by complement fixation and precipitation, using the corresponding polysaccharids as antigens.

Complement Fixation Test.—The serum was put in a series of 6 test tubes containing 0.2, 0.1, 0.05, 0.025, 0.0125 and 0.006 cc. of it respectively, made to 0.5 cc. with saline. Tube 1 was a control, containing serum only. To the remaining 5 tubes 0.5 cc. of the antigen was added. The complement consisted of 0.5 cc. of diluted guinea-pig serum, containing 2.5 lytic units. The mixtures were kept for 1 hour in the incubator, and then 1 cc. of the hemolytic system was added (0.5 cc. of a 5% goat erythrocytal suspension and 0.5 cc. of the anti-sheep serum having 3 times as strong a hemolytic power) incubation done for 2 hours more and the results were read.

The inulin, used as antigen in the complement fixation test was a 2% solution, the soluble starch a 1.5% solution, and the dextrine a 3% solution in saline. All were dissolved by heating. Each of these antigen-solutions (1.5 cc.) was tested and found to give no auto-inhibition.

EXPERIMENTAL

A. Inulin

Material for Injection.—Inulin was dissolved in saline to the proportion of 4% and heated at 70°C. Prior to injection, 0.8 cc. of the solution was mixed either with 0.2 cc. of the pig serum or with the same amount of the saline. The mixture was then put in the incubator for 1 hour. The controls received pig serum only, in the same dilution.

Doses and Number of Injections.—Each of the three mixtures described above was injected in a dose of 1 cc., 1.5 cc., 2 cc., 3 cc., 4 cc., 5 cc., and 6 cc., each dose being repeated.

Complement Fixation Test.—The normal serum often gave a very weak positive reaction. These positive sera, however, gave a negative reaction when they were inactivated by heating at 60°C. for 1 hour. They also gave a negative reaction when the mixture was allowed to stand at 0°C. for 1½ hours instead of at 37°C. before the hemolytic system was added. From these facts it will be seen that the positive reaction, which the normal sera gave, was non-specific in nature.

Results of complement fixation tests with the sera of injected rabbits are given in Table I.

From Table I, it will be seen that the sera of the rabbits, which had been treated either with inulin solution alone or with inulin-pig-serum mixture, all gave positive results. The control serum from the rabbit injected with the pig serum alone, gave a negative reaction.

Precipitation Tests.—These were carried out by both the mixing and the ring precipitation methods, but both yielded negative results.

Bunji Imai (8) has reported that by the complement fixation test he has demonstrated the antigenic power of inulin. He also obtained immune sera, which gave precipitation with a certain kind of inulin.

B. Soluble Starch

All the immunization experiments with a weak starch solution proved unsuccessful, but by the use of a strong one, the antibody formation was at last induced.

TABLE I

Results of the Complement Fixation Test with the Sera of the Rabbits Receiving Inulin Only

Rabbit No.	Serum (cc.)					
	0.2	0.1	0.05	0.025	0.0125	0.006
1	L	H	H	H	H	K
2	L	H	H	H	H	k
3	L	H	H	K	L	L
4	L	H	K	L	L	L
5	L	H	K	L	L	L
6	L	H	K	L	L	L

Results of the Complement Fixation Test with the Sera of the Rabbits Receiving Inulin Plus Pig Serum

7	K	H	H	K	k	L
8	k	H	H	H ·	K	k
9	L	H	H	H	K	L
10	H	H	H	H	K	L
11	H	H	H	H	H	K

H stands for inhibition, K or k incomplete hemolysis and L complete hemolysis in the complement fixation.

Material for Injection.—A 3% soluble starch saline mixture was heated and made into a pasty substance. It was used without any vehicle.

Doses and Number of Injections.—A daily injection with a dose of 0.5 cc. was given for 18 days.

Complement Fixation Test.—There was no case in which normal serum gave a positive result. After the completion of one course of injections, the sera of all the treated animals gave positive results, as shown in Table II.

Precipitation Test.—The tests all gave negative results.

Ken Nodzu (9) as well as B. Imai (8) have demonstrated the antigenic power of soluble starch by the complement fixation test.   Nodzu

TABLE II

Results of the Complement Fixation Test with the Serum of the Rabbits Receiving Soluble Starch

Rabbit No.	Serum (cc.)					
	0.2	0.1	0.05	0.025	0.0125	0.006
12	L	H	H	K	L	L
13	L	K	L	L	L	L
14	L	H	K	L	L	L
15	L	H	H	H	K	L
16	L	H	K	L	L	L

TABLE III

Results of the Complement Fixation Test with the Serum of the Rabbits Receiving Dextrine Only

Rabbit No.	Serum (cc.)					
	0.2	0.1	0.05	0.025	0.012	0.006
17	L	K	L	L	L	L
18	L	K	L	L	L	L
19	L	K	k	L	L	L
20	L	K	k	L	L	L
21	L	L	L	L	L	L
22	L	L	L	L	L	L

Results of the Complement Fixation Test with the Serum of the Rabbits Receiving Dextrine Plus Pig Serum

23	K	H	H	H	H	K
24	K	H	K	L	L	L
25	K	H	K	L	L	L
26	L	H	H	H	K	L
27	K	H	H	H	H	K
28	L	H	H	H	K	L
29	K	H	H	H	H	K
30	L	H	H	K	L	L

stated that he, like myself, obtained only negative precipitation tests, but Imai states that he obtained a weakly positive reaction.

C. Dextrine

Experiment 1

Material.—The dextrine used in my experiments was a white powder, which gave a purplish indigo color with iodine. It was insoluble except when heated; and therefore must have been amylodextrine. (For convenience sake it is stated as "D.I.") A 6% mixture of D.I. with the saline was made by heating at 70°C. The pig serum was added in the same proportion as in the case of inulin.

Dose and Number of Injections.—These were the same as in the case of inulin.

Complement Fixation Tests.—The normal sera gave positive reactions less frequently than in the case of inulin, and gave less marked reactions. By differential testings, the reaction was proved to be nonspecific.

The results of the complement fixation tests with the sera of rabbits treated with dextrine alone or with the mixture of dextrine and pig serum are shown in Table III. It will be seen that the serum of the rabbits immunized with the mixture of dextrine and pig serum gave a strong reaction, whereas those from animals receiving dextrine alone yielded doubtful reactions. The control serum from the rabbit injected with pig serum alone, gave a negative reaction.

Precipitation Reaction.—The results were entirely negative. Thus far, dextrine had induced antibodies only when injected in association with pig-serum. Because of the fact, however, that inulin and soluble starch gave antibodies even though injected without the addition of pig-serum, I next submitted animals to an increased amount and number of injections, in order to determine whether or not the addition of pig-serum is absolutely necessary to effect the antibody formation.

Experiment 2

Material.—The kind of dextrine and the concentration of its solution were the same as employed in Experiment 1.

Dose and Number of Injections.—18 injections with a dose of 5 cc. were given.

Complement Fixation Tests.—The results of the complement fixation tests made with the sera of the treated animals are shown in Table IV. It will be seen that dextrine when given by itself elicited antibodies.

Experiment 3

Material.—I used two different kinds of Merck dextrine, both being further decomposed than that of the previous experiments. One was a white powder given a purple color with iodine, and easily soluble. (For convenience sake this

lot is called "D.II.") The other was a yellow granular substance giving a red color with iodine and also easily soluble. (This is called "D.III.") D.III. was found to be erythrodextrine. In degree of decomposition, D.II. stood just between D.I. and D.III. The D.II. and D.III. specimens were dissolved in saline to a proportion of 6%, and injected into animals without the addition of the pig serum.

Amount and Number of Injections.—18 injections were given with a dose of 5 cc.

Complement Fixation Test.—All the sera gave negative results.

Experiment 4

Material.—A 6%, 10% and 15% saline solution of the D.II. and D.III. were prepared.

Amount and Number of Injections.—One course of treatment consisted of 5 injections each of 6%, 10% and 15% solutions in saline, the dose being 10 cc. The total amount of the injected material was, therefore, about three times as much as it was in the previous experiments.

TABLE IV

Results of the Complement Fixation Test with the Serum of the Rabbits Receiving Dextrine Only

Rabbit No.	Serum (cc.)					
	0.2	0.1	0.05	0.025	0.012	0.006
31	L	H	H	K	L	L
32	L	H	K	L	L	L
33	L	H	H	L	L	L
34	L	H	H	K	L	L

Complement Fixation Test.—The results of the complement fixation test with the sera of the animals treated with D.II. and D.III. were all negative. It may be inferred that these two kinds of dextrine were lacking in antigenic action.

From the results of my experimental immunization with dextrine, it might be concluded that the results of Imai, as stated above, who could not successfully immunize the animal with seven repeated injections of 5 cc. of a 6% dextrine solution as such or mixed with 0.5 cc. of the inactivated pig serum, were due either to an insufficient number of injections or the use of an unsuitable kind of dextrines.

Specificity of the Antibody

After heating the immunized sera at 60°C. for 1 hour, no changes in the complement fixation reaction occurred. Nor was the reaction

influenced by carrying out the first half of the procedure of the test at 0°C. for ½ hour. From these facts, it may be concluded that the reaction is of a specific nature.

In order to determine the specificity of the immune serum reaction against the above mentioned three polysaccharids, I carried out cross tests with them. In this experiment, I used a 1.5% solution of the three polysaccharids as antigen. The results are shown in Table V. Each gave a specific reaction.

TABLE V

Results of the Complement Fixation Tests for the Specificity of the Immune Bodies Derived from Polysaccharids

Antigen......................	Soluble starch				Inulin				Dextrine (D.I.)			
Amount of sera cc..............	0.1	0.05	0.025	0.012	0.1	0.05	0.025	0.012	0.1	0.05	0.025	0.012
Soluble starch immune rabbit serum:												
No. 15...............	H	H	H	K	L	L	L	L	L	L	L	L
No. 12...............	H	H	K	L	L	L	L	L	L	L	L	L
No. 16...............	H	K	L	L	L	L	L	L	L	L	L	L
Inulin immune rabbit serum:												
No. 11...............	L	L	L	L	H	H	H	K	L	L	L	L
No. 10...............	L	L	L	L	H	H	K	L	L	L	L	L
No. 5...............	L	L	L	L	H	K	L	L	L	L	L	L
Dextrine (D.I.) immune rabbit serum:												
No. 29	L	L	L	L	L	L	L	L	H	H	H	K
No. 30	L	L	L	L	L	L	L	L	H	H	K	L
No. 31...............	L	L	L	L	L	L	L	L	H	K	L	L

It may be noted here that the D.I. immune serum did not give any positive reaction to D.II. or D.III.

Very recently, Ken Nodzu (10) immunized an animal with starches from Indian corn, barley, wheat, potato, etc., and Yoshio Masuda (11) with those from two varieties of rice. Each of the immune sera reacted most strongly against that kind of starch which had been used as the antigen, while against others the reaction was very weak. The authors, therefore, insisted that they could immunologically differentiate starches, which it is very difficult to do by morphological features.

Fate of the Antibodies in the Animal Body

Blood was collected from the immunized rabbits at the end of every week and the complement fixation test was carried out with sera heated at 60°C. for 1 hour. The results are shown in Tables VI, VII and VIII.

TABLE VI

Results of the Complement Fixation Test with the Sera of Rabbits into Which Inulin Was Injected

Day of collection of blood	Results of the complement fixation with		
	Serum of Rabbit 10	Serum of Rabbit 11	Serum of Rabbit 5
1st week............	H H H K L L	H H H H H K	L H K L L L
2nd week............	L K k L L L	L H H H K L	L K L L L L
3rd week............	L K L L L L	L H H K L L	L L L L L L
4th week............	L L L L L L	L H H L L L	
5th week............		L H L L L L	
6th week............		L L L L L L	

TABLE VII

Results of the Complement Fixation Test with the Sera of Rabbits into Which Soluble Starch Was Injected

Day of collection of blood	Results of the complement fixation with		
	Serum of Rabbit 12	Serum of Rabbit 13	Serum of Rabbit 14
1st week............	L H H K L L	L K L L L L	L H K L L L
2nd week............	L H K k L L	L L L L L L	L K L L L L
3rd week............	L K L L L L		
4th week............	L L L L L L		

TABLE VIII

Results of the Complement Fixation Test with the Sera of Rabbits into Which Dextrine Was Injected

Day of collection of blood	Results of the complement fixation with		
	Serum of Rabbit 29	Serum of Rabbit 30	Serum of Rabbit 28
1st week............	K H H H H K	L H H K L L	L H H H K L
2nd week............	L H K L L L	L K K L L L	L H K L L L
3rd week............	L K L L L L	L L L L L L	L H K L L L
4th week............		L L L L L L	L K L L L L
5th week............			L L L L L L

It will be seen that the sera in the period following the immunization lost their strength rapidly, those of animals weakly immunized giving a negative result in a fortnight, while the strongly immunized gave one in 6 weeks.

Do the Polysaccharids Themselves Elicit the Formation of Antibodies?

The above mentioned polysaccharids gave rise to antibodies, even when unassociated with vehicle. But is it the polysaccharids as such that cause the antibodies to develop, or may a trace of proteins co-mingled in the materials play the part of vehicle? To settle the matter I have carried out an experimental investigation.

In the first place, I made Millon's and biuret tests upon the three kinds of polysaccharid solutions. They all gave a negative result. I then tried quantitative estimation of the nitrogen in the specimens after Kjeldahl's microestimation method. The results were that inulin contained 0.036%, soluble starch 0.017% and dextrine (D.I.) 0.019% of nitrogen.

Are the Nitrogen Contents Proteins?

In order to find whether the nitrogenous substances are proteins the following experiments were carried out:

To 200 cc. of a 2% hydrochloric acid solution 20 gm. of soluble starch was added, and the mixture was hydrolyzed by being boiled for 3 hours. The liquid was then neutralyzed and subjected to the iodine reaction test. It proved negative. It was, therefore, certain that all the starch had been converted into glucose. For the purpose of removing the salts and glucose, some of the liquid was then put into a bladder and left in running water for 48 hours. Then, the remaining liquid in the bladder was evaporated to 1/10 volume. The concentrate had a blackish brown color, one so dark that it was impossible to make color reaction tests with it. It was found, however, to contain 0.01% of nitrogen on quantitative estimation. The liquid gave also a positive reaction of Heller's ring test.

Another portion of the hydrolyzed solution, which had been neutralized, was allowed to evaporate to 1/10 without being dialyzed and a quantitative estimation was made of its nitrogen contents. It was found to contain 0.011% of nitrogen. This liquid also gave a positive result with Heller's ring test.

From the results of the above described experiments, it seems certain that the nitrogen found in the soluble starch solutions was protein.

Is the nitrogen, which is found in inulin or dextrine, of protein origin?

This theme remains to be studied. It will probably be hard to free these polysaccharids perfectly from nitrogenous substances.

From all the accessible data it might be inferred that it would be difficult to determine whether or not proteins play an important part as the vehicle in the immunization processes, but from the following two facts, proteins are suspected to be intimately related with the antibody production by polysaccharids.

1. Considering the connection between concentration, dose and number of injections of the used polysaccharid solutions and also the amount of the produced antibodies, it might be judged that inulin gives rise to antibodies comparatively easily. However, it is hard to obtain immunization, with soluble starch and dextrine. The nitrogen contents of the latter two is only about one half that of the first.

2. Comparing the results of the complement fixation tests with the immune sera of rabbits injected with inulin and dextrine with and without pig-serum, it will be seen that while there did not occur any differences with inulin, which is relatively rich in nitrogen, with dextrine, which is poor in nitrogen, the serum of the animal treated with the mixture of dextrine and pig-serum gave by far the stronger result. (See Tables III and IV.)

The facilities for successful immunization depend largely upon the natural characteristics of the materials injected and the nature of the animals, yet from the above mentioned two facts it may well be thought that there is an intimate connection between the production of immune bodies for polysaccharids and the association with them of proteins.

The possibility arises that the antibodies demonstrated by the complement fixation test, might have been directed against incidental protein. This is rendered rather unlikely by the following considerations.

1. As described above, the dialyzate obtained from the hydrolyzation products of the starch solution yielded an absolutely negative iodine reaction, but was positive to Heller's test. However, the complement fixation test made with this dialyzate and the anti-starch serum proved negative.

2. The polysaccharid solutions, which were used as antigens, gave no color reaction of proteins. Their nitrogen contents was at a

minimum and probably was not protein. Granting all of it to have been such, the total amount of nitrogen found in the material employed as antigen in the complement fixation test was extraordinarily small. On calculation the proteins in 0.5 cc. of the polysaccharid solutions, which I employed in my test, would be about 8/1,000,000 gm. in the starch specimens and 20/1,000,000 gm. in those of inulin. The real protein contents was doubtless but a fraction of these calculated protein contents. It is unlikely that these traces should produce such a remarkable complement fixation reaction as I have obtained.

CONCLUSIONS

1. By complement fixation tests, it has been clearly demonstrated that the sera of rabbits immunized with inulin, soluble starch and dextrine contain specific antibodies.

2. All these immune sera gave a negative precipitation reaction.

3. The kind of dextrine which has a construction very near to starch has an antigenic property, but those in a state of further decomposition do not give rise to antibodies.

4. All the three kinds of polysaccharids have power to produce antibodies without any vehicle. Dextrine is the only one of the three that gives rise to immune bodies more readily when pig serum is added to it.

5. Regarded as antigens, inulin stood first and soluble starch and dextrine next in order.

6. All three kinds of polysaccharids that were employed gave a negative protein color reaction. All of them, however, contained nitrogen. It has been proved that the large portion of the nitrogen contained in the soluble starch is derived from its protein contents.

7. It is suggested that in the production of immune bodies by these three kinds of polysaccharids, proteins might play the part of the vehicle. This is, however, still to be determined.

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THE ANTIBODY RESPONSE IN THE HUMAN BEING AFTER INJECTION WITH NORMAL HORSE SERUM

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During the course of an investigation of the antibody response in persons treated with immune sera (1–2), studies were carried out upon the sera of three patients who had received 50 cc. of normal horse serum by intramuscular or subcutaneous injection and where bleedings had been secured at close intervals. In all these cases, in contrast to those which had received immune sera, there was found a failure of antibody response and even a minor skin sensitiveness—in spite of the large dosage. Serum sickness had also failed to appear in these cases, and it was to this that the absence of circulating antibodies was attributed, since their production has been found, usually, to be correlated with the appearance of this phenomenon (1–4).

To test this assumption, sera were studied in cases of mild skin lesions receiving varying amounts of normal horse serum as a form of non-specific protein therapy. The study was carried out in the same manner as in that previously reported (1). The patients were bled prior to the injection and at various intervals, approximately the same as in the immune serum cases, thereafter; and exactly the same procedures were carried out in the determination of the various types of antibodies: precipitins, anaphylactic antibodies, reagins and the transferred guinea pig skin reaction.

A total of 91 sera from eighteen patients were thus studied. Normal horse serum, without preservative, obtained in four different lots from the pooled blood of six to eight normal horses was used. Skin tests to horse serum, in a dilution of 1:10, made before treatment, were uniformly negative. Two of the patients received 100 cc. by intravenous injection; two, 50 cc. by intramuscular; one, 50 cc. by subcutaneous; seven, 50 cc. by intravenous; five, 20 cc. by intravenous; and one, 10 cc. by intravenous. Serum sickness occurred in fifteen of the eighteen cases, (83%), in this group—an incidence about the same as that found in our series of immune serum treated cases.

TABLE I

Anti-Body Response in Cases Treated with Normal Horse Serum

Case No.		Treatment	Interval after treatment	Serum sickness	Skin reaction	Prausnitz-küstner	Guinea pig ear reaction	Anaphylactic antibodies	Precipitins
1.	I	10 cc., intra-venous	Before	—	Neg.	Neg.	±	—	—
	II	venous	14 days	—	Neg.	—.	Neg.	Neg.	Neg.
	III		21 days	—	Neg.	—	—	—	—
2.	I	20 cc., intra-venous	Before	—	Neg.	Neg.	Neg.	Neg.	Neg.
			10 days	Very mild					
	II		14 days		++	—	Neg.	—	1:10, +
3.	I	20 cc., intra-venous	Before	—	Neg.	Neg.	—	—	—
			5 days	Moderately severe, 3 days					
	II		9 days		±	Neg.	—	—	Neg.
	III		16 days		++	Neg.	++	Neg.	Neg.
	IV		23 days		++	—	Neg.	Neg.	—
4.	I	20 cc., intra-venous	Before	—	+	—	Neg.	Neg.	—
			10 days	Moderately severe, 3 days	+	Neg.	Neg.	Neg.	Neg.
	II		19 days		±	Neg.	Neg.	Neg.	Neg.
	III		28 days		+	Neg.	+++	Neg.	Neg.
5.	I	20 cc., intra-venous	Before	—	Neg.	—	Neg.	Neg.	—
			8 days	Marked, 4 days					
	II		22 days		++	Neg.	Neg.	Neg.	—
6.	I	20 cc., intra-venous	Before	—	Neg.	Neg.	Neg.	Neg.	Neg.
			10 days	Moderately severe, 3 days					
	II		14 days		+	Neg.	Neg.	Neg.	1:10, ±
	III		17 days		+	Neg.	—	Neg.	Neg.
	IV		21 days		++	Neg.	+	Neg.	Neg.
	V		28 days		++	Neg.	+	Neg.	Neg.
7.	I	50 cc., intra-venous	Before	—	Neg.	Neg.	Neg.	Neg.	—
	II	venous	7 days	—	Neg.	Neg.	Neg.	Neg.	—
			11 days	Severe, 5 days					
	III		12 days		++	Neg.	Neg.	Neg.	—
	IV		16 days		+++	Neg.	Neg.	Neg.	—
	V		21 days		+++	Neg.	Neg.	Neg.	—
	VI		24 days		+++	Neg.	Neg.	Neg.	Neg.
	VII		31 days		++	Neg.	Neg.	Neg.	Neg.

TABLE I—*Continued*

Case No.		Treatment	Interval after treatment	Serum sickness	Skin reaction	Prausnitzküstner	Guinea pig ear reaction	Anaphylactic antibodies	Precipitins
8.	I	50 cc., intra-venous	Before	—	Neg.	Neg.	Neg.	Neg.	—
			6 days	Marked, about 5 days					
	II		7 days		±	Neg.	+	Neg.	—
	III		20 days		++	Neg.	+++	Neg.	—
	IV		34 days		++	Neg.	—	—	Neg.
9.	I	50 cc., intra-venous	Before		Neg.	Neg.	±	Neg.	—
			Same day	Marked local reaction					
	II		7 days		Neg.	Neg.	Neg.	Neg.	Neg.
	III		14 days		±	Neg.	++	Neg.	—
	IV		18 days		±	Neg.	Neg.	Neg.	—
10.	I	50 cc., intra-venous	Before	—	Neg.	Neg.	Neg.	Neg.	—
	II		7 days	—	Neg.	Neg.	Neg.	±	—
			8 days	Severe, 4 days					
	III		14 days		+	Neg.	++	—	—
	IV		21 days		+	—	—	Neg.	—
	V		35 days		+	Neg.	Neg.	Neg.	Neg.
11.	I	50 cc., intra-venous	Before	—	Neg.	Neg.	Neg.	Neg.	—
	II		7 days	—	Neg.	Neg.	Neg.	Neg.	—
			10 days	Moderately severe, 8 days					
	III		14 days		±	Neg.	+	Neg.	Neg.
	IV		21 days		+++	Neg.	Neg.	Neg.	—
	V		29 days		++++	Neg.	Neg.	Neg.	—
	VI		39 days		++++	Neg.	Neg.	Neg.	—
12.	I	50 cc., intra-venous	Before	—	Neg.	±	±	Neg.	—
			6 days	Moderately severe, 2 days					
	II		9 days		+	Neg.	—	Neg.	Neg.
	III		16 days		+	Neg.	Neg.	Neg.	—
	IV		30 days		±	Neg.	±	Neg.	Neg.
13.	I	50 cc., intra-venous	Before	—	Neg.	Neg.	Neg.	Neg.	—
			6 days	Marked, 4 days					
	II		7 days		Neg.	Neg.	±	Neg.	Neg.

TABLE I—*Continued*

Case No.	Treatment	Interval after treatment	Serum sickness	Skin reaction	Prausnitz-küstner	Guinea pig ear reaction	Anaphylactic antibodies	Precipitins
13. III		9 days		Neg.	Neg.	+	Neg.	—
IV		14 days		++	Neg.	Neg.	Neg.	Neg.
V		17 days		++	Neg.	Neg.	Neg.	—
VI		27 days		++	Neg.	Neg.	Neg.	—
14. I	50 cc., intramuscular	Before	—	±	Neg.	Neg.	Neg.	—
		Same day	Urticaria and marked local					
II		7 days		Neg.	Neg.	+	Neg.	—
		10 days	Severe, 4 days					
III		15 days		++	Neg.	Neg.	Neg.	Neg.
IV		23 days		+	Neg.	++	Neg.	Neg.
V		30 days		++	Neg.	±	Neg.	—
VI		41 days		++	Neg.	Neg.	Neg.	Neg.
VII		53 days		++	Neg.	—	—	—
VIII		74 days		++	Neg.	—	—	—
15. I	50 cc., intramuscular	Before	—	Neg.	Neg.	±	Neg.	—
		Same day	Marked local					
II		9 days		Neg.	Neg.	+	Neg.	—
		10 days	Severe, 4 days					
III		17 days		++	+	++	—	1:10, ±
IV		23 days		+++	+	Neg.	Neg.	1:10, ±
V		30 days		+++	±	+	Neg.	—
VI		38 days		++	Neg.	+	Neg.	—
VII		56 days		++	Neg.	+	Neg.	—
16. I	50 cc., subcutaneous	Before	—	Neg.	—	—	—	—
II		1 day	—	Neg.	—	Neg.	Neg.	—
III		5 days	—	Neg.	—	Neg.	Neg.	—
IV		10 days	—	++	Neg.	±	—	—
V		21 days	—	—	—	±	+	—
VI		29 days	—	++	Neg.	±	Neg.	—
17. I	100 cc., intravenous	Before	—	Neg.	Neg.	Neg.	Neg.	Neg.
II		7 days	—	Neg.	Neg.	++	Neg.	—
III		14 days	—	+	+	Neg.	±	1:10, ±
IV		20 days	—	+++	++	Neg.	Neg.	Neg.
V		24 days	—	++	++	Neg.	Neg.	Neg.
VI		38 days	—	++	+	Neg.	Neg.	—
VII		52 days	—	+++	+	Neg.	Neg.	—

TABLE I—*Concluded*

Case No.	Treatment	Interval after treatment	Serum sickness	Skin reaction	Praus-nitz-küstner	Guinea pig ear reaction	Ana-phylac-tic anti-bodies	Precipi-tins
18. I	100 cc., in-travenous	Before	Immediate urticaria	±	Neg.	++	Neg.	Neg.
II		7 days		Neg.	Neg.	+	Neg.	Neg.
		11 days	Doubtful					
III		12 days		Neg.	Neg.	+	Neg.	—
IV		19 days		+	Neg.	+	Neg.	Neg.
V		26 days		++	Neg.	Neg.	Neg.	Neg.
VI		32 days		++	Neg.	—	—	—
VII		35 days		+	Neg.	Neg.	Neg.	Neg.
VIII		54 days		+	Neg.	Neg.	Neg.	Neg.

The results of the antibody studies of these sera are shown in Table I. When the antibody response in this group is compared with that in which immune sera were used, the almost complete failure of antibody response to the injection of normal horse serum is striking, even though in several instances the amount injected was about the same as that usually used for treatment with immune sera. When we consider the antibodies in particular we find that the precipitins were always lacking or doubtful; anaphylactic antibodies were not found in any case; reagins were demonstrated with very weak reactions in two cases, one of which was a skin case with eczema (possibly allergic); skin sensitivity, though present, was certainly less marked than in the former group; and the guinea pig ear-skin reaction, which in the immune serum group was usually the most consistently demonstrable of all the reactions used,—the first to appear and the last to disappear,—was entirely negative, or weak, at best. In general, the findings for this group, when summarized and compared with those for sera in immune serum treated cases, indicate an almost complete failure in (circulating) antibody response, when normal horse serum is used for treatment.

That this failure may not be charged to variations in the conditions of the two experiments, the following observations are offered: Several lots of horse serum were used during the experiment, obviating any special antigenic value that might be ascribed to the serum of an

individual animal; serum sickness similar in every respect to that occurring after immune serum injection occurred, usually in a more or less severe form, in as great a percentage of cases as in the first experiment; finally the cases for both groups were equally unselected.

This failure to find circulating antibodies to horse serum, as such, after treatment with normal horse serum may be seen as presenting certain obstacles to that theory of the mechanism of serum sickness which would correlate the clinical manifestations with the presence of circulating antibodies. It is to be noted however, that the studies, upon which these theories have been based have been carried out with various types of immune sera; no reference has been found in the literature as to the use of normal horse serum for such a study as is here given.*

The relative merits of the various theories as to the basic mechanism of serum sickness have been adequately reviewed by von Pirquet and Schick (4) and by Coca (6); it is necessary only to point out, in view of a more or less complete failure of normal horse serum to call forth the production of the antibodies responsible for the Prausnitz-Küstner reaction, precipitins, the passive transfer of the anaphylactic reaction, and the transferred guinea pig skin reaction—as shown in the protocol—that the theory of a basic antigen-antibody reaction in serum sickness is further questioned. Subsequent investigation, however, may afford an explanation of the parodoxical findings here presented.

SUMMARY AND CONCLUSIONS

After the injection of normal horse serum in the human being, serum sickness occurs even more regularly than in cases treated with the various immune sera, but this is not accompanied by the production, to any notable degree, of circulating antibodies of the various types that are regularly to be demonstrated after the administration of immune serum and its resulting serum sickness.

Since normal horse serum therefore appears to be weakly antigenic, and immune serum highly antigenic for the human being, one must

* Dr. Coca, in a private communication as to unpublished data of his study of serum sickness among Indians (5) following the use of normal horse serum, reports that he was unable to demonstrate precipitins at any time.

assume that this difference is the result of some alteration in its antigenic characteristics produced during the course of the immunization or of its preparation for use; or that the specific antibody which is responsible for the phenomenon of serum sickness has not yet been identified; or that this phenomenon is not in any way dependent on the presence of the various known antibodies to normal horse serum.

The authors wish to express their appreciation of Dr. Jay Frank Schamberg's courtesy in furnishing access to clinical material, and to the Mulford Company for their generous donation of the serum used in the experiments.

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THE EFFECTS OF CATHODE RAYS ON THE PROTEINS
OF SERUM

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Experiments conducted by Dr. Carrel in this laboratory revealed that chicken fibroblasts underwent a very characteristic change when cultivated in serum which had been submitted to irradiation by cathode rays. The cells resembled in appearance those of colonies cultivated in proteoses or peptones. Their cytoplasm was completely filled with granulations but, unlike degenerating cells, they maintained long, sharp, active pseudopods. They did not, however, undergo the rapid proliferation which is caused by partially hydrolyzed protein. On the other hand, they multiplied slightly more rapidly than control tissues cultivated in portions of the serum that had not been irradiated. These phenomena indicated that the cathode rays had produced certain chemical changes in the serum, probably in its protein constituents. The following study was made in an attempt to ascertain the nature of these changes.

The cathode rays were supplied by a Coolidge cathode ray tube,[1] operated at about 200 kilovolts (Tables I and II), and 0.2 milliamperes. The electrons did not penetrate more than a millimeter beyond the surface of the serum, so that the material was exposed either directly in the form of a thin layer, or in a cell behind a very thin mica window. In the latter case, much longer exposures were necessary, and the liquid was kept constantly stirred.

Fifteen cubic centimeter samples of chicken plasma were coagulated on Petri dishes of 180 sq. cm. area and exposed in a nearly vertical position 8 cm. from the window of the cathode ray tube. Exposures were made for 30 and 45 minutes. The serum was extracted from the irradiated plasma and also from control samples of coagulated plasma by grinding with sand. The cryoscopic points were determined and any necessary correction for evaporation was made. All samples were analyzed (1) for total nitrogen by the Pregl[2] micro method, (2) for amino nitrogen

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[1] Coolidge, W. D., *J. Franklin Inst.*, 1926, 202, 693.

[2] Pregl, F., Die Quantitative Organische Mikroanalyse. Berlin, 1923, 113.

by the Van Slyke[3] method, and (3) for non-protein nitrogen by precipitation with trichloracetic acid and determination of the nitrogen in the filtrate. There was a decided decrease in the concentration of protein nitrogen, the loss being from 32 to 42 per cent of the quantity originally present (Table I). There was a decrease in amino nitrogen corresponding to the loss of protein, and a small but significant increase in non-protein nitrogen of 15 to 42 mg. per 100 cc. (Table II). Since the protein which had disappeared did not appear in the non-protein fraction, it had evidently become insoluble and had been removed with the fibrin in transforming

TABLE I

Effect of Cathode Rays on the Concentration of Protein Nitrogen in Serum

Kind of serum used	Time of exposure	Voltage in kilovolts	Concentration of protein nitrogen			
			In control sera, mg. per 100 cc.	In irradiated sera, mg. per 100 cc.	Decrease	
					mg. per 100 cc.	per cent of original concentration
	min.					
Chicken serum from irradiated plasma						
1	30	210	474	273	201	42
2	45	210	399	243	156	39
3	45	195	664	454	210	32
Chicken serum irradiated as such						
	hrs.					
4	2½	160	555	510	45	8.1
Ox serum						
5	5	180	1,135	1,015	120	10.6
6	3	180	1,130	1,110	20	1.8
7	3	195	1,146	1,066	80	7.0
8	3	195	1,204	1,144	60	5.0

the plasma to serum. This supposition was substantiated in later experiments in which serum itself was irradiated.

A flat, vertical cell of about 20 cc. capacity was used for the irradiation of serum. The rays were admitted through a mica window 0.01 mm. thick and about 7 sq. cm. in area. Vigorous stirring was effected by means of a glass paddle. The time of irradiation varied from 2½ to 5 hours (Tables I and II).

Both ox serum and chicken serum were irradiated in the cell. The chemical changes observed were strictly analogous to those noted in the serum from the

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[3] Van Slyke, D. D., *J. Biol. Chem.*, 1912, **12**, 275.

irradiated plasma (Tables I and II).   During the course of the irradiation, a tough coating of yellowish-brown solid formed on the window of the cell.   It was insoluble in dilute acids and alkalies, unchanged by alcohol and ether, but dissolved in concentrated nitric acid.   The serum removed from the cell was distinctly opalescent, but no separation of solid material was obtained when it was centrifuged for a half hour, at 36,000 r. p. m. at 12 cm. radius.

The solid which collected on the window of the cell was dried and weighed. Analysis showed it to contain 11.8 per cent nitrogen.   The decrease of nitrogen in

TABLE II

Effect of Cathode Rays on the Concentration of Non-Protein Nitrogen in Serum

Kind of serum used	Time of exposure	Voltage in kilovolts	Concentration of non-protein nitrogen		
			In control sera, mg. per 100 cc.	In irradiated sera, mg. per 100 cc.	Increase, mg. per 100 cc.
	min.				
Chicken serum from irradiated plasma					
1	30	210	66.0*	108.0*	42.0
2	45	210	26.9*	42.1*	15.2
3	45	195	92.0	107.0	15.0
Chicken serum irradiated as such					
	hrs.				
4	2½	160	10.5*	14.7*	4.2
Ox serum					
5	5	180	43	72	29
6	3	180	59	79	20
7	3	195	45	96	51
8	3	195	46	77	31

* Precipitation by trichloracetic acid.

the total amount of serum irradiated was equal, within the limits of experimental error, to that which separated in the solid material.

In order to ascertain whether the cathode rays were affecting the albumin or the globulin of the sera, samples were analyzed for their albumin and globulin contents by precipitation with 22 per cent sodium sulfate at 33°C.   In the ox serum, there was an average decrease in albumin corresponding to 600 mg. of nitrogen per 100 cc., or 88 per cent of the albumin originally present.   In the globulin fraction, there was

an apparent average increase of 475 mg. of nitrogen per 100 cc. It could readily be shown, however, that this apparent increase in the globulin fraction was not caused by the formation of globulin, but rather by the production of denatured albumin which, like globulin, precipitates with 22 per cent sodium sulfate. This was demonstrated by a test on some crystalline egg albumin, about 40 per cent of which was denatured by heating at 40°C. In this sample of albumin, exactly the same quantity was precipitated by sodium sulfate as by adjusting to the isoelectric point of denatured albumin. In the chicken serum, there was a decrease in the globulin fraction as well as in the albumin fraction, showing that globulin also is converted to insoluble material.

Irradiation of solutions of pure proteins showed that the changes observed in the sera were properties of the proteins as such, and not dependent on the presence of other constituents of the serum.

Samples of crystalline egg albumin recrystallized three times, egg globulin precipitated twice with ammonium sulfate, and casein dissolved in 1 per cent sodium carbonate, were irradiated. In each case, a tough, insoluble deposit was formed on the window of the cell and a small amount of non-protein nitrogen was produced. About 92 per cent of the albumin was denatured. Fifty-nine per cent of the globulin and 11 per cent of the casein separated as insoluble material. When the albumin was irradiated in 0.9 per cent salt solution instead of water, more of the denatured material remained in solution. This fact explains the apparent increase of globulin in the irradiated sera, which was noted above. Further evidence of denaturation was given by a positive nitroprusside reaction in the irradiated albumin.

It is interesting to note that the chief effect of cathode rays on proteins is similar to that of ultraviolet light observed by Harris,[4] and of sunlight as observed by Young.[5]

Although the change of greatest magnitude brought about in the proteins by means of the cathode rays was that of denaturation, there was, as stated above, both in serum and in pure protein solutions a small increase in the non-protein nitrogen. In order to ascertain whether this should be ascribed to hydrolytic cleavage of the protein molecule or to some other type of change, an attempt was made to determine the nature of the products formed.

[4] Harris, L. G., *Proc. Roy. Soc.*, Series B, 1923, 94, 426.
[5] Young, E. G., *Proc. Roy. Soc.*, Series B, 1922, 93, 235.

The separation of proteins from their hydrolytic products depends upon their relative solubilities in various reagents. As shown by Hiller and Van Slyke,[6] sodium tungstate precipitates a large amount of the intermediate proteolytic products, such as the proteoses, while $2\frac{1}{2}$ per cent trichloracetic acid allows a much larger proportion of these to remain in solution. Unpublished experiments carried out in this laboratory indicate that $2\frac{1}{2}$ per cent trichloracetic acid as recommended by Hiller and Van Slyke,[6] and even 2 per cent trichloracetic acid as used by Wasteneys and Borsook,[7] precipitate not only the original protein, but also substances formed from it which do not possess all the properties of the original material. Since it seemed likely that any hydrolytic cleavage of the protein produced by cathode rays would result in large fragments of the protein molecule, the probability existed that such products would be precipitated with the protein by trichloracetic acid. An attempt was therefore made to see if removing the protein by coagulation with heat at its isoelectric point would give

TABLE III

Comparison of Non-Protein Nitrogen in Irradiated Sera as Determined by Trichloracetic Acid Precipitation and by Heat Coagulation

Precipitation by trichloracetic acid			Heat coagulation of protein at pH 5.6		
Control, mg. per 100 cc.	Experiment, mg. per 100 cc.	Increase on irradiation, mg. per 100 cc.	Control, mg. per 100 cc.	Experiment, mg. per 100 cc.	Increase on irradiation, mg. per 100 cc.
43	58	13	43	72	20
39	50	11	59	79	20
25	36	11	45	96	51

any further evidence of the formation of such protein-like degradation products by the irradiation.

Two cc. of the irradiated serum and its control were diluted to 20 cc. with water. The pH was then adjusted to from 5.5 to 5.6, and the solutions heated to 100°C. for 2 minutes. As 5.5 is the isoelectric point of serum globulin and 5.4 that of denatured serum albumin, this procedure seemed best adapted for removing the protein by coagulation. The solutions were filtered and the filtrates analyzed for nitrogen by the Pregl micro Kjeldahl method. The concentration of nitrogen in these filtrates was higher than that in the filtrates from the trichloracetic acid for both the controls and the irradiated sera, and, moreover, the difference between the irradiated and the controls was also larger (Table III). These results indicate that products are formed by irradiation which are thrown down with the protein when trichloracetic acid is used for precipitation.

These filtrates, and also the filtrates from the trichloracetic acid precipitation, were analyzed for amino nitrogen by the colorimetric method of Folin.[8] In both

[6] Hiller, A., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, 53, 253.

[7] Wasteneys, H., and Borsook, H., *J. Biol. Chem.*, 1924-25, 62, 1.

[8] Folin, O., *J. Biol. Chem.*, 1922, 51, 377.

cases, the filtrates from the irradiated sera contained a higher percentage of amino nitrogen than those from the corresponding controls. This difference amounted to from 1 to 3 mg. per 100 cc. It is evident, therefore, that a slight hydrolytic cleavage of the protein molecule takes place, although it is very small in comparison with the amount of protein denatured.

Tests were also made on the non-protein fraction of the irradiated sera for changes in the ammonia and urea concentrations. A slight increase in ammonia occurred, but no increase in urea. In some cases, the very small decrease in urea of 1 mg. per 100 cc. indicated that some of the ammonia might be derived from that source. When a 0.1 per cent aqueous solution of urea was irradiated for 3 hours, 11.4 per cent of the urea nitrogen was transformed to ammonia.

SUMMARY AND DISCUSSION

The effects of cathode rays on the proteins of serum appear to be (1) denaturation of a large proportion of the albumin and globulin with the formation of products that are soluble at the pH of the serum; (2) the production of a tough and exceedingly insoluble substance on the window of the cell where most of the absorption of electrons occurs; (3) a slight hydrolytic cleavage of the protein molecule producing a small quantity of products having properties so near to those of the protein that they are precipitated by trichloracetic acid but are not removed by coagulation at the isoelectric point; (4) the production of a small amount of hydrolytic products not precipitated by trichloracetic acid; and (5) the formation of a small amount of ammonia, part of which at least is derived from the urea in the serum.

It is interesting to note that these changes are such as would bring about exactly those effects on fibroblasts which were observed when cultures were grown in serum which had been subjected to cathode ray irradiation. The proteins of serum have a retarding effect on the growth of fibroblasts.[9] We might, therefore, expect their removal by denaturation and coagulation to result in the slightly larger growth which was observed. The production of SH groups in the denatured protein molecule would also tend to have a beneficial effect, as has been observed in experiments with denatured albumin. A concentration of protein split products equal to that in the irradiated sera has been observed to produce cells of characteristic appearance, full of cytoplasmic granulations and possessing long, active pseudopods, such as those noted in colonies cultivated in serum which had been subjected to cathode rays.

CELL PROLIFERATION RESPONSE TO SULFHYDRYL IN MAMMALS

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Studies made at this Institute during the past two years have brought out the fact that the sulfhydryl group is an essential stimulus to cell proliferation in simple types of plants and animals (root-tips and *Paramecium*) (1:2). The practical and theoretical bearings of this finding are clear. They have been briefly outlined in the papers cited and elsewhere (3:4). It remained to test whether the principle developed from the work with the simple forms would be demonstrable in more complex and highly developed species. Not that there was any doubt as to the essential validity of the principle, but it was felt that its exhibition in mammals would strengthen the theoretical position by extending the boundaries of support, and at the same time provide a basis for one phase of its practical application, namely, to the healing of wounds.

It was decided to combine these two aspects of the problem within a single bracket. Consequently thio-glucose was chosen as the compound to be tested. The choice was based on the fact that this substance contains the cell proliferation stimulating -SH group attached to the natural sugar glucose which presumably would provide an easily utilizable source of energy for the multiplying cells. Thio-glucose possesses other advantageous properties. Its sodium salt is fairly easily prepared in high degree of purity (5) in crystalline form. This is quite soluble in water and on neutralization with HCl yields the free -SH compound which is reasonably stable at a phosphate buffered pH of 6.8 if kept cool and in a dark place. The compound as made by Dr. Gerrit Toennies, chemist to the Institute, was used in a concentration of 1:10,000 S, in the experiments to be described.

Male albino rats of about 100 grams weight served as the test objects. The procedure was as follows.

The rat was anesthetized with ether or urethane and the hair removed from the back or abdomen by clipping and shaving. Two approximately circular pieces of skin, ¾ inch in diameter, were then cut out on opposite sides of the depilated area, taking care that no muscle or fascia was removed therewith.

Each wound was then covered with a small piece of gauze on which was placed a small wad of absorbent cotton. This dressing was covered with a square of thin rubber dam sealed with collodion to the body surface so that communication between the two wounds was prevented. A small hole cut in the dam over the cotton wad allowed the application of the test and control solutions to the two wounds respectively. The whole was held in place by an appropriate strip of adhesive tape encircling the body and suitably perforated over the dressings.

The test solution contained sulfur in 1:10,000 concentration as thio-glucose liberated by HCl from sodium-thio-glucose. This was buffered to a pH of 6.8 by 1.0 cc. Sorensen's phosphate mixture to 100 cc. solution. The control solution contained glucose, NaCl, and phosphate mixture in concentrations equivalent to those of the test.

Each of the two wounds was kept moist with one of the solutions by simultaneously saturating the dressing therewith every two or three hours for a period of 48 to 72 hours. At the end of this time the dressings were removed and the results recorded. Which solution contained the thio-glucose was unknown to us until this had been done, the information being solely in the hands of the chemists.

Three series of experiments were run with ten, eight, and seven rats respectively.

In the first series, eight out of the ten rats showed a distinct acceleration of wound healing on the side to which thio-glucose had been applied. In the second lot six out of the eight were similarly affected, and in the third six out of the seven. In the last two series the failures could be attributed to the fact that the rubber dam barrier between the wounds was not complete and intermingling of the solutions occurred.

The evidences of more rapid healing were as follows: More rapid contraction of the edges of the wound; lack of bleeding on removal of the gauze; obviously greater cellular growth over the surface of the wound; and in some cases inability to remove the gauze because of its interpenetration by the new growth.

The accompanying photograph demonstrates some of these points. The lower wound which had been treated with the control glucose

solution was raw and showed but little evidence of healing. The gauze though in close contact with the raw surface lifted off easily. The upper wound, to which the thio-glucose had been applied, was dry, the area was diminished, and the cells had so grown on to and within the meshes of the gauze that its removal without tissue destruction was impossible.

The advantage possessed by the sulfhydryl-treated wound was in general maintained, this coming to complete healing some days before that of the control side.

FIG. 1. Photograph showing accelerating influence of sulfhydryl on cell proliferation in the rat.

Like stimulation of cell proliferation in man has been obtained. A report of the clinical application of these findings to wound healing is being prepared by Reimann.

SUMMARY AND CONCLUSIONS

These experiments establish the fact that the sulfhydryl group is stimulative of cell proliferation in mammals as in lower organisms. The fact that the stimulation is exhibited in such a wide diversity of species including both plants and animals is justification for the belief that it is the expression of a fundamental biological phenomenon.

The literature and discussion of the subject are to be found in the papers cited.

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ANTIBODY AND AGGLUTININ IN PNEUMOCOCCUS PNEUMONIA*

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Though a number of investigators have studied the appearance of antibody and agglutinin during pneumococcus infection in man and in animals, it has seemed desirable to pursue the matter further. For this purpose, we have used blood obtained from patients with Types I, II and III pneumococcus pneumonia at the Massachusetts General Hospital and from the private practice of one of us. Through the kindness of Dr. H. A. Christian of the Peter Bent Brigham Hospital and Dr. W. H. Robey and his associates of the Boston City Hospital, we have also had the opportunity to investigate the blood of patients in these institutions. We are especially indebted to Dr. Maxwell Finland of the Boston City Hospital and to Dr. George Walker of the Peter Bent Brigham Hospital for obtaining blood from patients in these hospitals. We are indebted to Dr. Lloyd D. Felton of the Harvard Medical School for his kindness in furnishing virulent cultures of the pneumococcus and for many helpful suggestions.

Method

Tests for antibody were made by inoculating intraperitoneally each of 14 mice with 0.2 cc. of the patient's serum plus 0.3 cc. of one per cent peptone solution, and directly after this with dilutions of a highly virulent culture of pneumococcus of the same type as that found to be the cause of the pneumonia. For this purpose, seven dilutions of an eighteen hour beef heart broth culture of the organisms were made as follows:—1:100, 1:1000, 1:10,000, 1:100,000, 1:1,000,000, 1:10,000,000

and 1:100,000,000 and 0.5 cc. of each of these dilutions injected into each of two mice.

The tests for antibody were thus done in duplicate. In addition, no serum was given to two mice used as controls, one receiving 0.5 cc. of 1:10,000,000 and the other the same amount of 1:100,000,000 dilution of the culture. All mice were observed for 96 hours. We have regarded the survival of 1 mouse of the series inoculated with the patient's serum and organisms as an indication of protection. This seems reasonable in view of the death of the control mice and the fact that in all patients whose blood was tested before treatment within the first 5 days of the illness no protection was found. We realize a possibility of error in this, but such error would change the finding in only 3 out of 57 patients.

Tests for agglutinin were made with suspensions of living or dead homologous pneumococci at varying dilutions (1:2, 1:4, 1:8, 1:16, 1:20, 1:40, 1:80, 1:100, 1:200, 1:500) of the patient's serum according to the method of Arlyle Noble (*J. Bact.*, 14, 287, Nov., 1927), who uses small amounts of serum and concentrated suspensions of organisms. It was found that the tests were easier to read after a half hour of incubation, and this variation in the technique was made.

The blood of 57 patients was investigated for both antibody and agglutinin. On 46 of the series from two to fifteen tests were made. In the specifically treated cases the first sample of blood was taken before serum was given. Blood for subsequent tests on these cases was taken not sooner than eight hours after the administration of serum. Only 1 observation was made on 11 patients.

The results may be sketched as follows:—

Observations on Antibody in Pneumonia

Time of appearance of antibody during the disease

Our observations confirm those of other investigators in the finding that antibody in untreated cases appears at or about the time of the fall in the temperature. Of 49 patients to whom no serum had been given to the time the test was made none were found to have protection before the sixth day. Of 6 patients not treated with serum and tested at the time of the temperature fall only 1 failed to show antibody. The earliest time of appearance in the untreated group was the sixth day in 2 patients in whom the temperature fell within the next twenty-four hours.

In the treated group protective substances were frequently demonstrated after the use of Felton's antibody at a time in the course of the disease when protection would otherwise not be expected.

Behavior of Antibody during Convalescence and in Complicated Cases

Nineteen patients with antibody at the time of the fall in temperature were tested during convalescence. Protection was present in 8 from a few days to a week, in 2 into the second week, in 3 into the third, in 4 into the fourth, in 1 into the fifth and in 1 into the ninth week. In 11 of these cases no further tests were made. The remaining 8 showed that the antibody, previously present, was absent in 2 in the first, in 1 in the second, in 2 in the third, in 2 in the fifth and in 1 in the eighth week.

Four other patients who had had pneumonia 7 to 13 months previously, but had not been tested during the course of the disease, failed to show antibody.

In addition to the patients already referred to, 1 with unresolved pneumonia was found to have protection from the fifth to at least the forty-ninth day after the onset of the disease. His febrile course lasted 43 days. One patient with pneumonia and empyema was first tested 3 weeks after the estimated onset of the illness. At this time, he had antibody which persisted for at least four weeks longer. Another patient had empyema following pneumonia and had developed antibody when first tested, but died three days later of pneumococcus endocarditis, eighty-six days after the onset of the illness.

In our small series the duration of antibody after the fall in the temperature was variable. There appears to be no difference in its duration in those with and those without specific treatment. In the treated group, early or late treatment seemed to have no influence on its persistence.

The explanation of the disappearance of antibody in certain cases and its persistence in others is not clear. The presence of an unresolved pneumonia, or such complications as empyema or pneumococcus endocarditis, may have been responsible in certain cases, but in others no explanation is apparent.

Relation of Antibody to the Outcome

Of 35 patients who showed antibody at some time during the course of the disease, 29 recovered and 6 died. Of the 29 who recovered,

antibody was present at the time of the fall in the temperature in all. Of the 6 fatal cases, all were treated, none before the fifth day. One had empyema and pneumococcus endocarditis and another cardiac and renal complications. There were no obvious complications in the remaining four. All 6 had demonstrable antibody to within thirty-six hours of death.

Of 6 patients without protection during the course of the disease 5 died and 1 recovered. None were specifically treated. The patient who recovered failed to show antibody during the last three days of the disease and when tested five times during a subsequent period of twelve days.

Observations on Agglutinin in Pneumonia

The behavior of agglutinin was much the same as that of antibody. When present during the disease in patients without specific treatment it was first demonstrated at the time of the crisis or lysis. It was frequently demonstrated in specifically treated patients earlier than would otherwise be expected. It was present in all but 4 of 27 patients at the time of the fall in temperature. During the post-febrile period agglutinin was usually found to persist for a shorter period than did antibody.
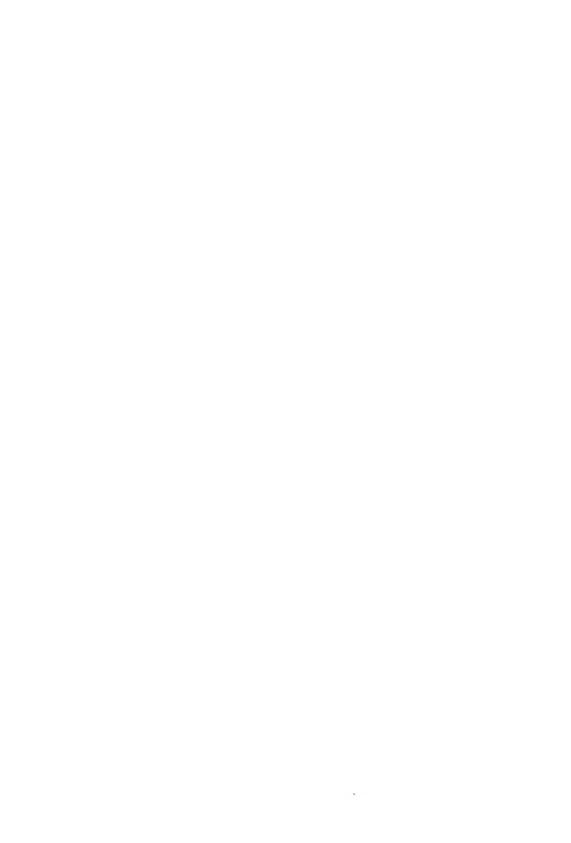
Value of Tests for Agglutinin as an Indication of the Presence of Antibody during the Course of the Disease

Inasmuch as tests for agglutinin in the patient's blood are not difficult to perform the question arises whether the presence or absence of agglutinin may be useful as an indication of the presence or absence of antibody and the control of dosage of specific serum. In an attempt to answer the question, we have correlated the findings with respect to these two substances in 32 patients. Agglutinin and antibody appeared simultaneously in 8. Agglutinin preceded antibody in 5 cases, in 3 of these by one day and in 2 by two days. In 2, agglutinin was present without antibody at any time during the course of the disease. Of the remaining 17 patients, in 9 antibody preceded agglutinin by one day in 3, by two days in 1. by three days in 4 and by four days in 1. In 8 patients antibody was present without demonstrable agglutinin in repeated tests.

Agglutinin may be present even in high titer without any demonstrable antibody and conversely there may be abundant protective substances without demonstrable agglutinin.   In 7 of 32 patients the presence of agglutinin if interpreted as an indication of the presence of antibody, would have led to an erroneous conclusion regarding treatment.   Agglutinin, therefore, is unreliable as a control of dosage. The clinical aspects of the case seem to us a better means of judging the desirable amount of serum and the frequency with which it should be administered.

CONCLUSIONS

1. Protective substances and agglutinins were not demonstrated in the blood of patients with pneumonia untreated with serum before the fall in the temperature by crisis or lysis.

2. Protective substances and agglutinins were frequently demonstrated in serum treated patients during the course of the disease.

3. Protective substances and agglutinins were usually present at the time of the temperature fall in patients untreated with serum and persisted for days or weeks thereafter.

4. Protective substances have an important bearing on the outcome. A large proportion of those with protection recover and a large proportion without it die.

5. Agglutinin and antibody may appear simultaneously or one may precede or be present without the other.

6. Tests for agglutinin are unreliable as a control of dosage of specific serum.

ETIOLOGY OF OROYA FEVER

XVI. Verruga in the Dog and the Donkey

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Plates 21 to 23

In the older literature on verruga[1,2,3,4] are found statements that
in endemic centers the disease may sometimes occur spontaneously in
domestic animals, particularly horses, mules, dogs, and pigs. That
distinct differences in the appearance of the nodular lesions existed,
or were thought to exist, among these animals, is apparent from the
fact that they were used as criteria for describing certain types of
lesions in man; for example, the deep-seated, bulky lesions were, and
still are, called "mular" or "mulaire," whereas the superficial ones
were sometimes referred to as "verrugas de caballo" (verruga of
horses). The variation in appearance of the nodules seems to have
been accepted by tradition, since careful descriptions of verruga in
animals cannot be found, and actual records of experimental inocula-
tions in the closely related species of horses, mules, and donkeys are
apparently very rare. The experiment of Ribeyro, Mackehenie, and
Arce,[5] in 1913, with a donkey, is the only one of the kind on record.
The animal was inoculated by scarification of the skin at the inner
canthus of the eye, and on the nose, with the "pulp" of a verruga
nodule obtained from a patient. After 21 days the skin at the sites
of inoculation showed papules 3 to 4 mm. high, which increased only
very slightly in size during the next 6 days. Then they began to
regress and after 11 days had entirely disappeared. The reaction
seems to have been a very mild local one.

In their attempts to find an animal susceptible to *Bartonella bacilli-
formis*, Noguchi and Battistini[6] tried ringtail, rhesus, Java, and green
monkeys, dogs, rabbits, mice, rats, and guinea pigs, and found that

results were definite only in the *rhesus* monkey. Later, experiments were started (Noguchi) in dogs, horses, and donkeys, the results of which are recorded below. In the dog mild verruga was induced, in the horse and in burros the results were negative so far as concerned the production of true verrugous skin lesions; in the horse and in one burro there was induration at the sites of inoculation, which lasted 3 to 4 weeks, but *Bartonella bacilliformis* was not recovered from the lesions. Further experiments, with donkeys, have been more successful.

Dog 1, female, inoculated March 23, 1926, intradermally, on eyebrows and abdomen, with (a) a mixture of 4-weeks old cultures, 1st and 2nd generations, grown on blood agar slants and leptospira medium, from the blood of *M. rhesus* 14,[7] a chimpanzee,[7] and *M. rhesus* 18,[8] and (b) a saline suspension of nodular tissue excised* from *M. rhesus* 18. 2 cc. of the culture mixture were injected intravenously. Small nodules developed after 8 days at the sites of culture inoculation, but none at the sites inoculated with nodule suspension. Four days later one of the nodules had reached a diameter of 0.5 cm. and was excised.* *Bartonella bacilliformis* was recovered in culture from a suspension of the tissue. Blood cultures made at this time were negative.

Microscopic examination of nodular tissue: In the spaces among the thick collagen fibers of the dermis there is a proliferation of endothelial cells. This process is chiefly diffuse, but small foci also are found. There is also a considerable polynuclear exudate and a moderately extensive plasma cell exudate. Except for the latter findings, the lesion is histologically similar to the lesions produced in monkeys by *Bartonella bacilliformis*.

Colt, about 3 years old. Inoculated March 30, 1926, intradermally, at two sites on the left flank, with mixed cultures (from blood agar slants and semisolid leptospira medium) of *Bartonella bacilliformis*. There was no change in the animal's temperature, which was normally 100.4°F. (38°C.), and blood culture was negative 8 days after inoculation. After 20 days there was definite induration at the sites of inoculation, which disappeared within the next two weeks. A second inoculation was made at 4 sites on the neck, on April 22, with the material inoculated on that day into Donkey 1. After 4 to 6 days there was marked induration at the two sites of culture inoculation, but none at the sites inoculated with nodular tissue and blood, and there was no fever.

Donkey 1. Inoculated April 22, 1926, at 4 sites on the left flank and 3 on the left side of the neck, with (a) 15-day culture from blood agar slant, 5th generation from M. rhesus 7,[8] (b) 14-day culture on leptospira medium, 1st generation from *M. rhesus* 29,[9] and (c) saline suspension of nodular tissue from *M. rhesus* 18,[8]

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* All operations were performed under ether anesthesia.

and (d) citrated blood from *M. rhesus* 25.[8]   8 cc. of the mixture were injected into the jugular vein. There was no change in the animal's temperature, which remained at about 100°F., and blood culture was negative 4 days after inoculation. At this time there was marked edema along the neck and flank and at other points remote from the sites of inoculation. After 8 days there was definite induration at the inoculated sites, and one of the "nodules" was removed for culture and section. *Bartonella bacilliformis* was not recovered from the suspension of tissue, nor were the organisms seen in the sections. Histologically, however, the section has the appearance of that from a very early verruga lesion. Several minute foci of endothelial cell proliferation are present in the deep portions of the dermis, and there is a tendency to the formation of sheets of cells and capillaries. A moderate polynuclear infiltration is also present.

In the second donkey the inoculation gave rise to edematous areas remote from the areas of inoculation, but there was very little reaction at the inoculated sites.

Donkey 2, inoculated Nov. 30, 1926, intradermally, on the left side of the neck, and also intravenously (4 cc.), with 8-day cultures of *Bartonella bacilliformis* grown on blood agar plates. There was no change in the animal's temperature, which remained slightly below or above 38°C. Rather large, fluctuating swellings appeared within 48 hours on various parts of the body, one on the side of the back, and others on the flank and abdomen. Blood culture was negative 72 hours after inoculation, and also after 20 days. The swellings were still present 2½ months later, and one was punctured; the aspirated fluid was sterile. A piece of tissue, removed from another "nodule" at this time for section, was histologically negative. Six weeks later a saline suspension of tissue from one of the "nodules" was inoculated into *M. rhesus* 30, with negative results, both as to blood culture and local lesions. The animal's appearance was nearly normal 284 days after inoculation. Its weight at this time was 440 lbs. Reinjection of the animal 8 months later, that is, a year and a half after the original inoculation, with live cultures, produced the same reaction as before, and in this instance there were generalized small scaly eruptions all over the body, which persisted about a week.

The results in Donkey 2 were difficult to interpret, and two more donkeys were inoculated, one with killed cultures of *Bartonella bacilliformis*, and the other with a filtrate of living cultures. The reaction in these two animals was much the same as in Donkey 2. In the animal receiving the filtrate (Donkey 3) the swellings appeared within 72 hours after inoculation (Fig. 1); in Donkey 4, inoculated with heat-killed cultures, they did not develop fully until about 10 days after inoculation. In both, however, they persisted for several weeks.

The study of sections made from the swollen areas yielded no explanation of the phenomenon which occurred in these two animals.

Donkey 3, weight about 500 lbs., injected April 4, 1928, intravenously, with filtrate (Berkefeld V) of 14-day cultures of *B. bacilliformis*. The temperature was 102.4°F. late in the day of inoculation, but thereafter remained a few tenths of a degree below or above 100°. Large raised areas developed in 72 hours, as in Donkey 2 (Fig. 1). Blood culture was negative 9 days after inoculation. Control *rhesus* monkeys (injected with the same material, intravenously and intradermally) developed no skin lesions, and blood cultures were negative. Cultures of the inoculated material also proved its sterility.

Donkey 4, 650 lbs., injected April 16, 1928, intravenously, with cultures of *Bartonella bacilliformis* killed by heating 30 minutes at 60°C. This material was proved sterile by culture tests and animal inoculation. The animal's hide was not perfectly smooth, and the natural prominences were outlined with a skin pencil before inoculation. 10 days after inoculation this animal was found to have developed the same swellings as Donkeys 2 and 3, but they were less marked. The appearance of the animal 25 days after inoculation was similar to that of Donkey 3. A piece of tissue removed from a swelling on the left side of the animal 8 months after inoculation was found on microscopic examination to consist solely of fat tissue.

The donkeys used in the first four experiments were of the small (440–650 lbs.) light gray variety commonly known as the "burro." The fifth donkey, in which definite verruga lesions were produced (Fig. 3), was a larger animal (800 lbs.) and brown in color. So far as can be ascertained, however, the species is the same.

Donkey 5. Weight 800 lbs. Five intradermal injections were made, on Jan. 5, 1929, with a mixture of living cultures of the 5 strains of *Bartonella bacilliformis* on hand in the laboratory,[10,11] two into the skin of the left flank, two on the right ear, and one on the left ear. 10 cc. of the same mixture were injected intravenously. The material produced, on injection into the skin of the ears, swellings about 1 cm. in diameter, which did not subside but slowly increased in size, so that after 6 days they were 2 cm. in diameter and 12 mm. high (Fig. 3). They were dome-shaped and slightly fluctuant. The skin over them remained intact and was of the normal black color of the donkey's ear. The sites of inoculation on the flank secreted a clear serous liquid for a few days, without papule or nodule formation, and then completely healed.

The animal continued to receive intravenous injections of mixed strains of living cultures of *Bartonella bacilliformis*, 20–30 cc. at each dose, twice a week for 14 weeks, the idea being to produce, if possible, a strong local and general reaction, in the hope of obtaining an immune serum for use in other experiments.

After the nodules on the ears had reached a diameter of 2 cm. they remained stationary for a period of 3 weeks, after which they gradually became smaller until at 72 days after inoculation they were invisible.   Blood cultures were made repeatedly throughout the period of observation and yielded uniformly negative results.

One of the nodules on the right ear was aspirated on the 22nd day after inoculation and slightly less than 1 cc. of turbid liquid obtained which proved by culture tests to be sterile.   Both nodules on the right ear were punctured three days later, but no liquid was obtained from either.   The nodule on the left ear, similar in all respects to those on the right ear, was then removed under novocaine anesthesia. A portion was used for inoculation of a *rhesus* monkey, and another portion for histological study.

Animal passage.   M. *rhesus* D-1 was inoculated intradermally and by scarification on Feb. 16, 1929, with a saline suspension of a part of the nodule removed from Donkey 5.   After an incubation period of 9 days typical nodules (Fig. 4) developed at the sites of inoculation, and blood culture made the following day was positive for *Bartonella bacilliformis*.   Nineteen days after inoculation the nodules were mature, the area inoculated by scarification showed fully developed lesions, and after 51 days recovery was complete.   The disease ran a typical course.   The culture of *B. bacilliformis* obtained from the blood of this animal and a subculture from it, on intradermal inoculation into M. *rhesus* D-2 and D-3 produced typical reactions (Figs. 5 and 6).

Histological examination   (*Figs. 7 and 8*).   The tissue removed from the ear of Donkey 5 consisted of the covering epidermis, a narrow rim of normal skin encircling the nodule, and a superficial portion of the nodule itself.   The latter, irregularly marked off from the rest of the tissue, was soft, gray with a faint pink tinge, and about 4 mm. thick at the center, tapering off gradually in thickness toward the edges.   The tissue was fixed in Regaud's fluid, and sections were stained with hematoxylin and eosin and with Giemsa's solution.

The hematoxylin and eosin sections, examined with the naked eye, show a thin normal layer of epidermis covering a layer 1 to 1.5 mm. thick of apparently normal dermis.   Beneath this is a collection of small round bodies, closely packed and tending here and there to become conglomerate.   Each of these individual nodules, varying from 1 to 3 mm. in diameter, stands out from the rest of the tissue by its deeper hematoxylin stain.   Together they comprise the nodular tissue proper.

Microscopically the minute nodules which make up the large nodule are in general fairly distinct from one another, but in places they tend to fuse.   They are all very densely infiltrated with polynuclear leucocytes, which somewhat obscure the underlying structure by their presence.   However, numerous slit-like spaces of variable length are seen within the minute nodules, lined with large, oval, flat cells with clear oval nuclei.   These cells resemble endothelial cells. Some of the spaces contain serum; others are empty.   With higher magnification

there are seen larger collections of these endothelial cells forming sheets or running in bands, and having a tendency to form blood-containing capillary-like structures.

The overlying epidermis and the narrow zone of dermis, with its numerous hair follicles and sweat glands, are normal. Though there is no distinct capsule, these tissues are separated sharply from the collection of minute nodules beneath. The surgical line of excision passes through the nodular material.

These findings show that the unit of the pathological process situated in the deep portions of the dermis is a minute nodule, from 1 to 3 mm. in diameter. The units may be discrete or tend to fuse. Each is composed of a considerable proliferation of endothelial cells, arranged in sheets, bands, and tubes. Superimposed, there is a very marked polynuclear infiltration, as was found in the section of nodular tissue from Dog 1.

SUMMARY AND CONCLUSIONS

In the experiments here reported, definite verruga lesions, in which the presence of *Bartonella bacilliformis* was established by culture or by passage to *rhesus* monkeys, were produced in a dog and in a donkey by inoculation of cultures or monkey passage strains. The reaction induced in these animals was entirely local, however; blood cultures were sterile. Histologically, the lesions produced were similar to those obtained in monkeys by inoculation of *Bartonella bacilliformis*, except for the presence of a marked polynuclear leucocytic exudate.

In another donkey a lesion histologically suggestive of verruga was produced, while in one donkey and a horse the results of inoculation were negative or indefinite.

The intravenous injection of a filtrate or of heat-killed cultures of *Bartonella bacilliformis* into two donkeys was followed by the appearance of large, soft, subcutaneous swellings, on various parts of the body, not resembling in any way verruga lesions.

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EXPLANATION OF PLATES

PLATE 21

FIG. 1. Donkey 3, 72 hours after intravenous injection with a filtrate of cultures of *Bartonella bacilliformis*, showing the large swellings which arose on the side.

FIG. 2. Photograph of the same animal, taken before inoculation. For comparison with Fig. 1.
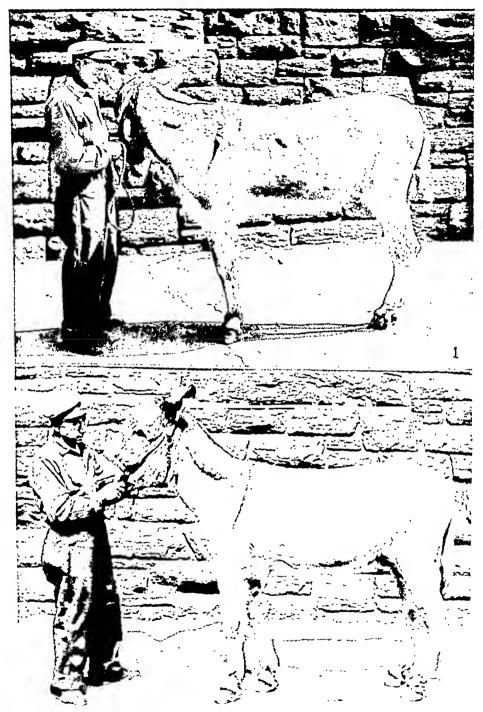
PLATE 22

FIG. 3. Appearance of nodules on right ear of Donkey 5, 39 days after inoculation. The nodule on the left ear had been partly excised 14 days previously, and the wound was in process of healing.

FIG. 4. *M. rhesus* D-1, showing verruga nodules on abdominal wall 10 days after inoculation with emulsion of nodule from ear of Donkey 5.

FIG. 5. *M. rhesus* D-2, 31 days after inoculation with culture of *Bartonella bacilliformis* from blood of *M. rhesus* D-1.

FIG. 6. *M. rhesus* D-3, 22 days after inoculation with subculture of *Bartonella bacilliformis* from blood of *M. rhesus* D-1.

PLATE 23
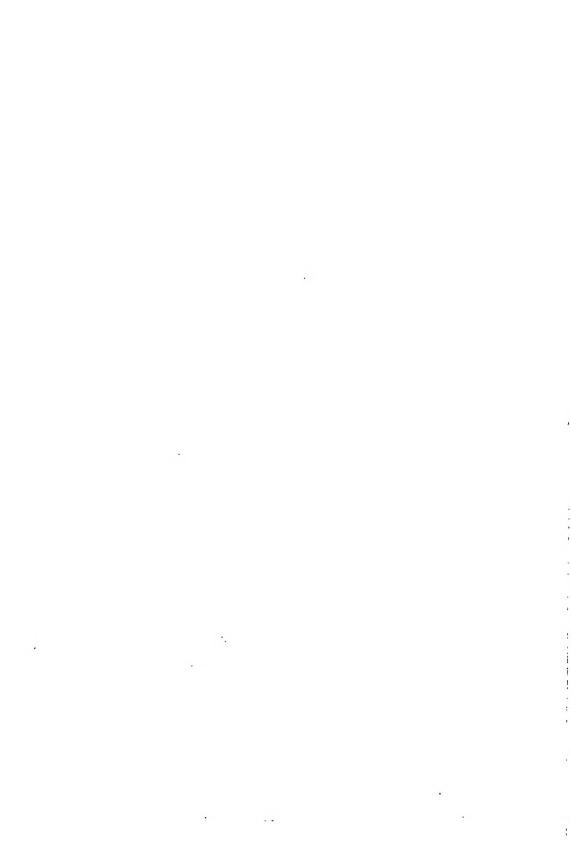
FIG. 7. Low power magnification ($\times$ 105) of section from nodule on ear of Donkey 5, showing one unit of the lesion.

FIG. 8. Higher magnification ($\times$ 600) of section from same nodule, showing proliferation of endothelial cells, lymph and blood spaces, and polynuclear infiltration.

PLATE 21

1

2

PLATE 23
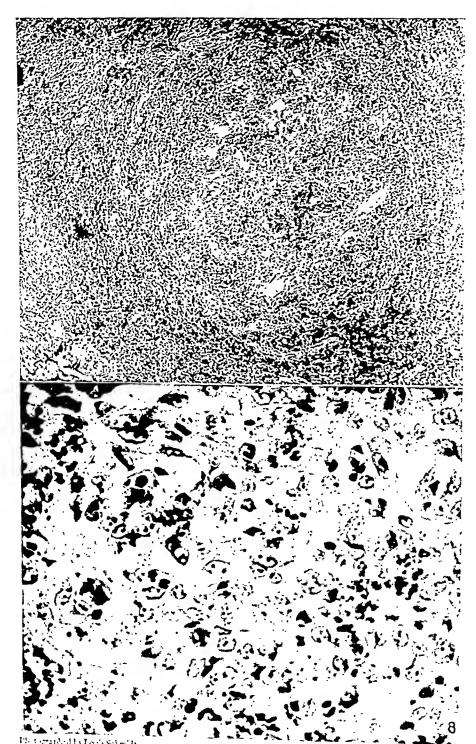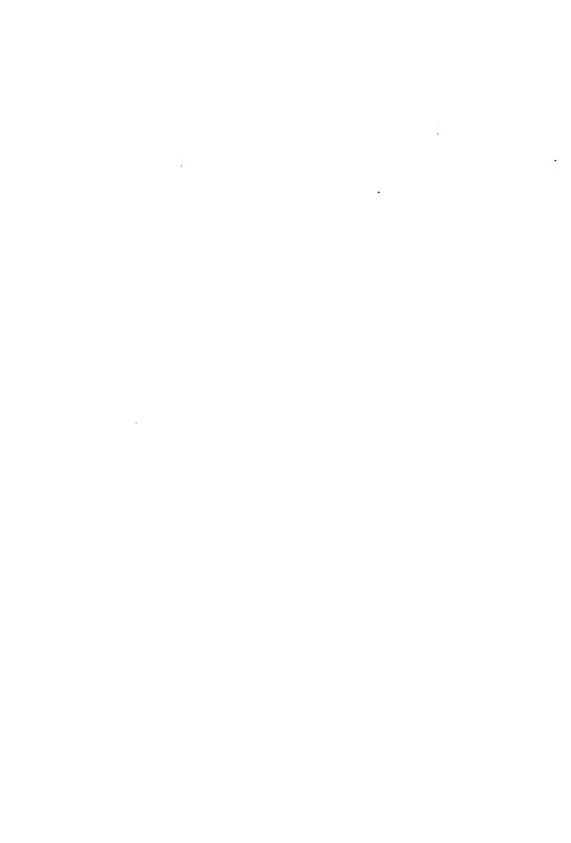
DIFFERENTIATION OF HEMOLYTIC STREPTOCOCCI OF HUMAN AND OF DAIRY ORIGIN BY METHYLENE BLUE TOLERANCE AND FINAL ACIDITY

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Although hemolysis on the blood agar plate was formerly considered a property peculiar to streptococci from pathogenic sources, it is now recognized that the same property is also possessed by many non-pathogenic strains. Means of distinguishing hemolytic strains that are pathogenic from those that are non-pathogenic is especially important in the sanitary examination of dairy products. Previous studies (1–9) on streptococci have dealt with the properties of hemolysis, final acidity, and reduction of dyes, but the correlation of the results has been made difficult by the fact that the different workers have had different interests. The medical bacteriologist has usually studied strains isolated from infections and has emphasized hemolysis or acid production without regard to dye reduction. The agricultural bacteriologist on the other hand has studied non-pathogenic or saprophytic strains isolated from milk products and has emphasized acid production or dye reduction without regard to hemolysis.

The present investigation was planned to attempt to correlate these differential characters previously used in their respective fields by medical and agricultural bacteriologists. In spite of the fact that no new methods were introduced, the results are of new interest: (1) the collection of hemolytic streptococci included both pathogenic strains representative of those associated with human disease and saprophytic strains representative of the normal flora of milk products; (2) the three tests (hemolysis, final acidity, and dye reduction) are applied at the same time to strains from the different sources.

Final Acidity and Source of Strains. The 138 strains of hemolytic streptococci included in our tests may be separated on the basis of their final hydrogen ion concentration into the two main groups described by Ayers (1) and by Avery and Cullen (2): those having a final acidity range of pH 5.3 to 5.0 represent the low acid group; the others with a range of pH 4.5 to 4.0, the high acid group.

TABLE I

Hemolytic Streptococci

Source	Number of strains
Low acid producing strains	
Sputum, pneumonia	6
Blood, septicemia, meningitis, osteomyelitis	7
Pleural fluid, empyema	19
Lung, pneumonia	12
Throats, pneumonia, measles	43
Pus, abscesses, cellulitis, scarlet fever	4
Cows' udders	4
Total	95
High acid producing strains	
Cheese	21
Butter starter	1
Milk (pasteurized 1, certified 2)	6
Udders	14
Cow feces	1
Total	43

Table I summarizes the grouping of the strains on the basis of final acidity of glucose broth cultures. All the strains from human sources fall in the group of low acid producers. While 4 of the strains included in the low acid producing group were isolated from the udders of cows, two of them were identified as belonging to the human type and were known to be responsible for two separate epidemics of septic sore throat. Hence, all but two of the 95 strains in the low acid

producing group were definitely associated with human infection.   In contrast to this, all of .the 43 strains producing high acidity had been isolated either from bovine sources or from dairy products.

Methylene Blue Reduction

All strains both of the low and high acid producing groups were tested for their capacity to reduce methylene blue in milk.

Three different concentrations of the dye were used: 1 to 20,000, 1 to 10,000 and 1 to 5,000.   Methylene blue milk was prepared by adding a sterile 0.4 per cent aqueous solution of medicinal methylene blue (Merck's) to sterile fat-free milk in amounts sufficient to give the desired final concentration.   Three tubes of methylene blue milk, containing respectively 1 to 20,000, 1 to 10,000 and 1 to 5,000 concentrations of the dye, were inoculated with 0.1 cc. of 24 hour broth culture of

TABLE II

Reduction of 1:5000 Concentration of Methylene Blue in Milk by High and Low Acid Producing Strains

Final acidity	Methylene Blue Reduction	
	Strains not reducing	Strains reducing
Low acid producing group...............................	94	1
High acid producing group...............................	19	24

the strain to be tested.   The inoculated tubes were incubated at 37°C. and observed for dye reduction over a period of seven days.

The tests with 1 to 5,000 concentration of methylene blue furnished the most definite distinction.   As shown in the summary (Table II), 94 of the 95 low acid producing strains failed to reduce methylene blue.   (Ninety-one of these low acid producing and non-dye reducing strains were isolated from human source).   On the other hand, the high acid producing strains, all of which were isolated from dairy sources, can be separated into two groups; strains which reduced and those which did not reduce the dye.   It is important that 14 of the 19 high acid producing strains that failed to reduce the methylene blue had been isolated from udders of cows; and that 21 of the 24 high acid producing strains that reduced the dye were from various kinds of cheese.

Bactericidal Action of Methylene Blue

Negative subcultures were obtained in tests of viability of the streptococci introduced as the test inoculum in the cultures in which no reduction of the dye had occurred. The apparent absence of living streptococci indicated that failure to reduce the dye in the preceding tests represented a lack of tolerance to methylene blue on the part of the non-reducing strains. Apparently in the unreduced state, the methylene blue either prevented the growth of the non-reducing strains (bacteriostatic action) or destroyed their life (bactericidal action).

In order to obtain further information on the influence of methylene blue upon the viability of the streptococci a constant amount (0.1 cc.) of broth cultures of reducing and of non-reducing strains was inoculated into milk containing a known concentration of the dye; plate cultures were made after different intervals of time. The original number of streptococci added to the methylene blue milk was known to be quantitatively sufficient to establish the growth of the more delicately growing strains in the medium containing no methylene blue.

The results of the typical experiments summarized in Table III show that approximately 90 to 97 per cent of the non-reducing streptococci were killed by 10 minutes exposure to a 1:5000 concentration of methylene blue in milk. Different strains of the non-reducing group varied somewhat in the time required to kill 100 per cent of the cells; with some strains, complete sterility was obtained within 30 to 60 minutes but with others a few viable cells persisted for several hours; in all cases, the cultures were completely sterile within 48 hours.

DISCUSSION

A collection of hemolytic streptococci from both human and dairy sources was studied in order to determine if the combined use of the properties of dye reduction and of final acidity would be of value in the differentiation of hemolytic strains of diverse origins.

Different concentrations of methylene blue were tried but it was found that a solution of 1 to 5,000 in milk gave the most clear cut separation of the strains into dye-sensitive and dye-tolerant groups. This concentration of the dye not only prevented growth but was

actually bacteridal to the dye-sensitive strains. The dye-tolerant strains grew in the presence of and reduced a 1:5000 concentration of methylene blue. Of the 113 dye-sensitive strains, 108 were isolated from human or bovine sources and in many instances were associated with infectious processes in man or cattle. The other five strains were from samples of fresh and certified milk and were probably of udder origin (hemolytic udder streptococci are more commonly found in freshly drawn and certified milk than in ordinary market milk where the more resistant varieties of milk streptococci predominate (7, 9)). In contrast to the direct animal origin of the dye-sensitive hemolytic

TABLE III

Hemolytic Streptococci; Bactericidal Action of 1:5000 Methylene Blue Milk

Period of Exposure to Methylene Blue 1:5,000	Number of viable organisms per cmm.			
	Strains not reducing Methylene Blue		Strains reducing Methylene Blue	
	Strain "2"	Strain "4"	Strain "P"	Strain "M"
minutes				
0	2,200	3,500	2,500	3,000
5	460	770	2,400	2,700
10	40	320	2,000	2,800
15	9	300	3,000	3,600
20	1	210	4,500	6,000
30	0	60	6,000	7,100
60	0	25	11,000	13,000

streptococci, the 25 dye-tolerant strains with only two exceptions[1] were isolated from pasteurized milk, cheese and commercial butter starter.

When both the methylene blue and the final acidity tests are applied to hemolytic streptococci from human and dairy sources, three groups are distinguished. First, a group of low-acid-producing strains that are sensitive to methylene blue; this corresponds to Avery and Cullen's low-acid-group from human sources. Second, a group of high-acid-

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[1] Although one of these strains was isolated from a human throat and the other from a cow's udder, their presence in these locations might have been due to their introduction from saprophytic sources; e.g., recently ingested food in the case of the throat strain, or the cow's bedding in the case of the udder strain.

producing strains that are sensitive to methylene blue, these are most commonly derived from the normal or diseased udder. Third, a group of high-acid-producing strains that are tolerant to methylene blue. This last group, the dye-tolerant and high-acid-producing hemolytic streptococci are different from the high-acid-producing but dye-sensitive strains included in Avery and Cullen's group of "bovine" strains. The latter are most frequently of udder origin and are often associated with bovine mastitis; their presence in dairy products is for the most part limited to fresh raw milk for they are relatively infrequent in older market milk or in stored dairy products like butter or cheese. The dye-tolerant group on the other hand are in general much more resistant to unfavorable conditions than are most hemolytic streptococci and consequently are found in cheese or other dairy products that have been subjected to storage or other processes which tend to kill off the dye-sensitive strains.

The grouping of streptococci upon this basis, while not an attempt at a systematic classification of the *Streptococcus* genus, is of interest from two different points of view. The results as a whole indicate, that with hemolytic streptococci there is an apparent relation between lack of tolerance to methylene blue and tendency toward parasitism; and that all three tests, namely hemolysis, final pH, and methylene blue reduction should be applied in the study of streptococci of disputed etiological and epidemiological significance.

SUMMARY

A grouping of 138 strains of hemolytic streptococci based on differences in dye-sensitiveness and in final hydrogen-ion concentration of cultures is presented. Three groups are distinguished; (1) human parasitic strains, defined by a final pH range of 5.2 to 5.0 and by failure to reduce methylene blue (1:5000) in milk, (2) bovine strains parasitic in the udder, characterized by a final pH range of 4.5 to 4.2 and by failure to reduce methylene blue (1:5000) in milk, (3) saprophytic strains, characterized by a final pH range of 4.5 to 4.2 and by ability to reduce methylene blue.

Methylene blue was bactericidal for the strains of hemolytic streptococci that fail to reduce it, but neither bacteriostatic nor bactericidal for the strains that caused its reduction.

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STUDIES OF TISSUE MAINTENANCE

III. Persisting Bloodlessness after Functional Ischemia

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When the circulation reenters a part that has been briefly deprived of blood there ordinarily develops in it an active hyperemia, as every student of vascular physiology knows. But if the deprivation has endured for a somewhat longer time the happening is diametrically different. As the present paper will show, an obdurate local vaso-constriction has in some way been invoked, one so effective that the tissue involved remains closed off from the circulation for a greater or less period even though the flow through neighboring regions of the same sort is unusually good and the systemic blood pressure above the original level.

Persisting ischemia of the sort here described can readily be demon-strated with the aid of highly diffusible vital dyes, in animals with blood bulk reduced by bleeding or by solutions inducing anhydremia. Under such circumstances a patchy ischemia of the superficial tissues develops after some minutes, one so complete that the patches become pronouncedly acidotic, and even highly diffusible vital stains (phenol red, brom phenol blue, Patent Blue V) fail to enter them (1). If the blood bulk be restored soon after these patches have developed they rapidly disappear; but when they have endured for a little while the restoration has merely the effect of accentuating them through the passage of additional stain into their surroundings, and an hour or more may elapse before, by a gradual coloration, they are lost in the general hue.

Experiment 1.—With barium sulphide the hair was carefully removed from the body and thighs of a well nourished and vigorous, white female cat. No cutaneous inflammation ensued during the next few days. The animal was kept in a well-warmed room. For twenty-four hours prior to operation it was given no food,

but was allowed water. Under ether the trachea was cannulated with a Y tube for the purposes of anesthesia; the right carotid was connected with a mercury manometer for blood pressure readings; and the left axillary artery and a vein of the left foreleg (radial branch of the cubital) were cannulated, for bleeding and the injection of solutions respectively. All this took 9 minutes. The cat was then laid upon its back on an electrically warmed pad. The limbs assumed natural positions. The carotid blood pressure was constant at 142 mm. Hg.

Now 30 cc. of blood was taken from the axillary artery in the course of 3 minutes 15 seconds, and 16 minutes later 17 cc. more (in 3 minutes), and after the lapse of another $10\frac{1}{2}$ minutes 8 cc. more (in 2 minutes). In this way the carotid blood pressure was reduced to 74 mm. 31 minutes from the beginning of the depletion an injection of phenol red was made. The cat weighed 2240 gm. and the warmed 4 per cent dye solution (at pH 7.4 and isotonic with the blood) was given in the ordinary proportion of $3\frac{1}{2}$ cc. per kilo, 8.25 cc. being gradually run into the vein during 80 seconds. After the injection the pressure did not improve, but tended to fall as in other animals bled to the limit and injected only with fluid which would not remain in the vessels. Three minutes later, when the pressure was only 60 but the breathing good, the lips, gums, and conjuctivae had become deep red, with an orange cast suggestive of acidosis, whereas the trunk and thighs showed merely a few orange patches widely separated by pallid unstained regions. By this time a normal cat would have been brilliantly red.

The blood, as taken, had been defibrinated with care so that as little as possible should be lost. It was now aerated, warmed and restored to the animal by successive injections. A mere $4\frac{1}{2}$ cc., given 3 minutes after the dye was all in, brought the pressure to 110 millimeters and 15 cc. 10 minutes later carried it to 150, that is to say above the initial level. Despite this recovery the staining scarcely progressed; and two further injections of all the remaining blood (22 cc. in 1 minute 45 seconds and $9\frac{1}{2}$ cc. in 35 seconds) were made 18 and 20 minutes respectively after the dye injection. The carotid pressure reached 160 and remained at that figure for the next three quarters of an hour. The heart beat violently and the arterial pulsations were accentuated. After the return of the blood the deeply stained conjunctivae, gums and lips practically at once became red without trace of orange—between *jasper red* and *eugenia red* (Ridgway) (2)—showing that the slight general acidosis had disappeared. Over the trunk and thighs, however, the changes were very gradual. Large areas were still unstained 3 minutes after the final blood injection, and where the dye penetrated it had a hue between *ochraceous orange* and *ochraceous buff*. Little by little the coloration extended, but even after half an hour there were still many unstained patches such as are ordinarily associated with a diminished blood bulk (3). The areas to which the phthalein had originally been distributed were now turning red whereas those into which it penetrated later were orange. These various local differences caused the body surface to appear piebald with orange, red and white. Forty-six minutes after the last return of blood there were no longer any unstained areas but a pronounced

spotting of pale orange upon red persisted. The orange patches were now small, only ½ to 1 cm. in diameter.

To hasten the disappearance of the patches 40 cc. of warm Ringer's solution was injected intravenously 47 minutes after the last return of blood. The injection took 2 minutes. The blood pressure, which had been at 158, fell transiently but recovered to 152. Within the next twenty minutes all the orange spots disappeared.

In this experiment the patchy ischemia induced by the bleedings lasted at least half an hour after the blood had been restored and very probably for more than an hour, if one can judge from the persistence of areas of local acidosis that had been slowly colored by diffusion inwards from the well-stained tissue round about. The preliminary bleedings had been done gradually and at intervals, in order to allow opportunity for readjustments within the organism, and more especially for the passage of fluid out of the tissues into the vessels to make up the blood bulk. As Starling has shown (4), compensatory changes of the latter sort are practically at an end within 5 minutes after a single brisk bleeding, and subsequent bleedings do not elicit them in any important degree. The depletion was pushed very nearly to the limit, as indicated by the tendency of the blood pressure to fall after it had been accomplished, despite the introduction of dye dissolved in a salt solution. The inability of salt solutions to restore the blood pressure on such extreme occasions is well recognized. Only 51 cc. of blood was returned as compared with 54 cc. taken, but the said 54 cc., as drawn from the vessels, consisted in part of tissue fluid; for, as is well known, the last blood removed at even a rapid bleeding is dilute as compared with the first. It follows that the actual quantity of blood taken was less than our figure would indicate. The circulatory state after the reinjection supports this view. It was one of evident plethora, the heart beating violently, the pulsation of the arteries unusually prominent, and the veins engorged.

The quantity of phenol red injected into the depleted animal was not reduced from that which would have stained a normal cat evenly and deeply. The blood bulk, though, had been reduced by nearly half at the time of the dye injection. The question comes up of whether under such conditions the persistent patching may not have been caused by an untoward influence of the dye. True, previous experi-

ment had repeatedly shown that an acidotic patching indicative of local ischemia develops when the vital staining precedes the bleeding (5), which last removes much of the dye still in circulation. But this does not mean that the patches would have persisted had the blood been restored to the body. To determine what influence, if any, the dye had in the persistence of the patching was not difficult.

Experiment 2.—A vigorous, male, white cat of 2240 gm. was prepared like that of Experiment 1, save that it was fasted for only 16 hours prior to operation. The same cannulations were performed, under ether, in 19 minutes. The carotid blood pressure was found to vary between 140 and 145 mm. of mercury. A bleeding of 38 cc. in 120 seconds was now carried out, and others followed of 12 cc. in 70 seconds, 15½ cc. in 90 seconds and 6 cc. in 45 seconds, 14½, 23½ and 51½ minutes respectively from the beginning of the first. The carotid pressure was in this way brought down gradually to 84 mm. Hg. The body surface had become notably pallid, with suggestions of even paler blotches here and there. The animal was still in excellent condition, stirring under the light ether. The blood pressure tended to fall after the last bleeding. It decreased to 74 mm. Hg in a period of 9 minutes, when the reinjection of blood was begun, the animal being in good condition at this time. Less than 2 cc. had been lost in defibrination but 8.4 cc. more was withheld, since the intention was to throw that much phenol red solution into the circulation, and the avoidance of a sudden plethora seemed desirable. All the rest of the blood, aerated and warmed, was introduced by way of a foreleg vein in the course of three minutes, and was followed immediately by the 4 per cent phenol red solution (during 65 seconds). The blood pressure rose to 148 and fell again slowly to 130. The tip of the nose and the gums became red but the superficies of the trunk and thighs colored very slowly, and where the phthalein entered it was at first orange, not red. Large patches remained wholly uncolored. 5 minutes after the dye injection the regions between these patches had for the most part changed from orange toward red, showing that the local acidosis was being dissipated; and the dye was slowly extending into the margins of the uncolored regions as a narrow zone of orange. After another half minute the remaining 8.4 cc. of blood was returned to the body. The pressure rose to 158 and soon to 168, near which high level it long remained. The coloration continued strikingly various. 7 minutes after the final injection of blood there were still many irregularly distributed, uncolored patches 1½ to 2½ cm. in diameter, of serpiginous outline. They were scattered thickly upon a background of varied hues, from *carrot red* to *old rose,* and at their periphery one could see a narrow zone, or edging, of *ochraceous buff.* The marked increase in the blood pressure facilitated the passage of the stain into the tissues where circulation was taking place, with result that 11 min. after the last blood injection the patches were even more pronounced than before because of the deeper red of the background. The mucous membranes of the mouth were now *eugenia red,* and the pads of the paws pink,

evidence that there was no blood acidosis; but the hue of the stained superficial tissues had some orange in it here and there. After 14 minutes many of the spots were disappearing; and the general hue was *light jasper red.* After 18 minutes stain had entered all the remaining patches, coloring them an orange buff. The blood pressure had now fallen to its initial level, 140 mm. Hg. Some of the orange spots were still to be discerned 55 minutes after the last injection of blood; but they had gone after another 34 minutes, when the surface color was everywhere the same, a hue between *eugenia red* and *old rose.* The blood pressure meantime had varied between 140 and 166. The ether was purposely kept very light.

These observations in which phthalein was injected after the blood had again been returned make plain the fact that the obdurate ischemia cannot be due to an influence of the vital dye. It is no artefact. That the bloodless state existed prior to the injection of phthalein was clear, not alone from the failure of the latter to enter large areas here and there, but from the hue it took on at the edges of these areas, a hue indicative of preexisting local acidosis such as develops only when ischemia has endured some time (6). There were vague indications of a special patching of the pallid skin even before the return of the blood. The intensification of the patches after the final blood injection, which resulted from a deeper staining of the tissue between them, is no unique phenomenon. We have regularly observed it, and,—as one would expect,—even more pronouncedly, when the blood that was withdrawn and returned to the body contained dye.

Prior to a consideration of the causes for the persistent bloodless state the circumstances of its occurrence will be more nearly dealt with. Those leading to the initial patchy ischemia have been described in several previous papers (7). The patches develop in compensation for a reduced blood bulk, however brought about; and they appear only when the reduction has been considerable. The greater the depletion, the larger are the bloodless areas; and if it persists unrelieved they increase in size by peripheral extension. In animals gradually depleted by induced anhydremia they appear before any noteworthy reduction of the general blood pressure occurs; but in hemorrhage cases, they are best seen when the blood pressure has been substantially lowered. They occur in both urethanized and etherized animals but develop also in those bled under local anesthesia, a relatively great depletion being required to cause them in these last. They occur when the skin is kept warm artificially as well as when it has grown cold owing to deficient blood flow.

The persistence of the patching after return of the blood to the body is dependent, like its initial occurrence, on the degree of the depletion and its duration.   The more the blood bulk is diminished, and the longer the elapsed period afterwards, the more obdurate does the patching prove.

Experiment 3.—A thin, white, female cat, with a few gray markings, previously depleted and fasted for 24 hours, was operated upon under ether to cannulate the trachea, right carotid artery and left jugular vein.   The animal weighed 1950 gm. The operation took 15 minutes.   The initial blood pressure was irregular varying from 120 mm. Hg to 150 mm.   With the animal on its left side in an unconstrained position 56 cc. of blood was removed at three bleedings, by way of the cannulated carotid, in the course of 40½ minutes, reducing the blood pressure to 38 mm. at which low level it remained, until,—after 45 minutes in all,—7½ cc. of phenol red solution was injected, in 100 seconds.   The animal appeared *in extremis* after 47½ minutes; and 2½ cc. of the heparinized blood was reinjected.   Artificial respiration was necessary for a few breaths.   It was given with the Gates' pump (8).   Then the pressure rose to 50 mm. and natural breathing was resumed.   After a further 2 minutes 3 cc. more of blood was injected, merely sufficient that is to say to maintain the animal.   The vital staining was for some time nearly negligible except that the lips and gums, which had appeared bloodless, promptly became red after the injection of the phthalein; but little by little color crept in, and 12 minutes after the last blood injection (or 15 minutes after the phthalein) the surface was strikingly variegated with orange to red areas betwixt large unstained patches. The pressure had reached 60 mm. Hg.   Now 3 cc. more of blood was given, and 7½ minutes later all the rest (in the course of 4½ minutes) except 9 cc.   The pressure at once rose to 152 millimeters and the veins became notably distended, yet four minutes later the mottling had not altered.   Thereafter the red between the patches became accentuated and these slowly turned orange with dye entering them, and eventually they merged with the red of their surroundings.   The last of them was not gone for about an hour.   To the blood as it was withdrawn salt solution, containing 10 mg. of heparin (Hynson, Westcott and Dunning) in every cc. was added in the proportion of 1 cc. for 15 cc. of blood.   What with this addition, the amount of fluid returned to the body, though 9 cc. less than the total of blood plus heparin, was only 5 cc. less than the total of blood removed.   A large experience with the anticoagulant enables us to say that it does not of itself induce local ischemia.

In this experiment the animal was bled to the limit and the blood was only very gradually returned to the body after the injection of phenol red.   At first the dye largely failed to enter the superficial tissue, and where it did so later the color indicated tissue acidosis.   Needless to say the very low blood pressure will amply account for these find-

ings. Later on, when the unstained patches were smaller and well demarcated, all of the blood except 5 cc. was gradually returned to the body. Although the blood pressure mounted far higher than before the bleedings, and there was a manifest plethora with engorgement of the veins, the spotting persisted for an hour. These results and those of Exp. 1 may be contrasted with the following:—

Experiment 4.—A well nourished, white female cat of 2540 gm. was prepared and operated upon as in the case of Exp. 1, except that the removal of fur had been done with the razor and the initial fast had lasted only 18 hrs. The cannulations, under ether, took 18 minutes. Thereafter the animal was bled 62 cc. in 54 minutes. The blood pressure was reduced from 170 mm. Hg merely to 100 mm. Pallid blotches on a generally pale skin became visible over the animal's right side—which was uppermost; and $19\frac{1}{2}$ minutes after the last bleeding $9\frac{1}{2}$ cc. of phenol red was injected (in 65 seconds). 4 minutes later the body surface was a brilliant pink variegated with numerous, scattered white patches up to 2 cm. in diameter. The return to the body of all the defibrinated blood was begun 11 minutes after the dye injection and completed $3\frac{1}{2}$ minutes later. The carotid pressure rose to 180, at which level it remained, and there was the usual evidence of a plethora. Within a minute the animal had stained much more deeply, and the spots stood out in startling contrast; but they soon began to turn pink and within thirteen minutes had disappeared. The cat was by now of a hue deeper than *old rose.*

In this instance although much blood had been taken the carotid pressure remained fairly high and the blood was returned to the body after only a short interval. The ischemic patches, while well defined, had not endured long, for no acidosis had developed in them. They stained pink, not orange, and quite rapidly.

To these contrasted findings may be added others of similar import in rabbits. Very deep urethane anesthesia so impairs the circulation that patching results, but ether does not do this unless pushed close to the lethal limit (9). In the tests now to be described the animals were bled with the aid of a local anesthetic, novocaine.

Experiment 5.—A white, female rabbit of 2400 gm. shaved over the trunk and thighs 9 days previously was fasted 24 hours, and under ether the right common carotid was brought to the surface and cannulated. The vessel was kept shut by a special device made of rubber tubing (10), the pressure of which could be relaxed at will and bleeding accomplished with the animal sitting naturally. The incision was swabbed with novocaine and covered with a wet dressing. When the animal was again up and about, after 63 minutes, novocaine was re-applied

locally and the depletion begun. During the course of an hour and five minutes 76 cc. of blood was removed—at three bleedings—and at once defibrinated. Immediately afterwards the animal appeared in good condition, sitting in the normal posture; but it was found collapsed and apparently moribund 18 minutes later. The reinjection of all of the blood by way of an ear vein, and removal to a very warm room, benefitted it but little. Eight minutes later, at the time when 9 cc. of phenol red was given, it was still pallid and appeared unconscious, but after another eleven minutes it got to its feet. The superficial staining at this time was localized to localities where large vessels entered the skin (popliteal space, regions just back of the axilla and over the sacroiliac joint). Here the hue was orange. The animal was sitting up and in fair condition 51 minutes after return of the blood, yet the skin still showed completely unstained areas on a ruddy to orange ground. Along the ridge of the back was a well demarcated orange stripe. The general condition continued to improve, but 79 minutes after the blood injection the patches previously unstained were still to be discriminated, though now *coral red* on a *jasper red* background. At this time 7.2 cc. of warmed 4 per cent brom phenol blue was injected in 60 seconds. Most of the surface of the animal colored as rapidly as if the animal had been normal, becoming a deep plum color within three minutes, but very little of the dye had entered the patches at the end of this time. After a further 4 minutes, though, they were no longer to be discerned as such, having merged in the general hue.

The introduction of a second dye into this animal brought out the fact that there was still some persisting local ischemia $1\frac{1}{3}$ hours after the blood had been returned to the body. During much of this period the general circulatory state had been poor. But the rapid distribution of the second dye everywhere in the superficial tissue except in the regions of ischemia clearly proved that these latter were not due to a faulty general condition.

In a second rabbit the phenol red was injected before the bleedings had all been wholly accomplished, with result that depletion was less drastic. The only patching obtained was one traceable to local pressure differences. Like the ischemia unlocalized by any such factor it persisted after return of the blood.

Experiment 6.—A male, white rabbit of 2175 gm. was employed which had been epilated over the trunk and thighs with barium sulphide some days previously. No inflammation of the skin had ensued. The animal was fasted for 24 hours, and then, under ether, the left common carotid was cannulated and obstructed with the same device as in Exp. 5. Prior to closing the incision the tissues were swabbed with novocaine. Fifty-four minutes after the operation had been completed the animal had recovered from the ether and was in excellent state. Now it was bled

28½ cc. in 75 seconds, and 30 minutes later another 21½ cc. in 110 seconds.   Thereafter the skin was pallid but still warm, and not blotched.   After the lapse of a further 32 minutes 12 cc. of blood was taken in 70 seconds.   As each lot was obtained it was defibrinated.   Twelve minutes later 8.5 cc. of 4 per cent phenol red was injected into an ear vein in 60 seconds.   This caused no symptoms, and the animal, which sat quiet, colored slowly and evenly.   So well had the depletion and staining been withstood that eighteen minutes later the rabbit was sniffing about the laboratory floor as if nothing had happened.   The general color was now *old rose* save for large patches over the ridge of the backbone and the projection of the knees where the hue was yellower.   Since no patching developed in the next twenty minutes a further bleeding of 15½ cc. in 3½ minutes was carried through. Thereafter the patching over back, sides and haunches became more pronounced, *light coral red* as compared with *light jasper red* elsewhere.   The animal retained the sitting posture but appeared listless.   The entire quantity of blood, 77½ cc., was now reinjected in 185 seconds, thirty-three minutes after the final depletion. The general condition immediately became excellent.   Yet the patches persisted unchanged during the next ten minutes, though the red about them became deeper, approximating *eugenia red*.   After another seven minutes those over the knees had disappeared and the one along the backbone was fading at the edges.   Twenty-four minutes after the return of the blood the color was everywhere the same, slightly deeper than *old rose*.   Decolorization, which now began, proceeded swiftly and so evenly as not to suggest that there had ever been patching.   The rabbit was *alizarin pink* one hour and forty-four minutes after the return of the blood and still faintly pink three hours after.   It was then discarded.

In this instance the removal under local anesthesia of about half of the calculated blood volume did not suffice to bring about a general patching, though the circulation was sufficiently cut down over back and knees for some local acidosis to develop there, as the indicator showed.   The abnormal state of affairs was rendered more pronounced by a further bleeding which the animal stood well.   And now when all the blood was reinjected and the general condition became excellent the local acidotic patching failed to disappear for a considerable time. That its obduracy was not due to factors effective under normal conditions was shown by the course of the eventual decolorization which took place at the same rate in the regions previously patched as it did elsewhere.

It has been our frequent observation that local pressure conditions of no moment ordinarily will lead in the depleted animal to a localized ischemia indistinguishable, save in situation and in the extent and shape of the patches, from that occurring elsewhere over the body (11).

Exp. 5 yielded an example of the sort, in the patching over the ridge of the backbone, while in Exp. 6 all of the patching was of such type. The ischemia tends to persist like that appearing elsewhere on the body and not evidently referable to any local pressure differences. We have enlarged the observations upon it because of the physiological problem presented by the persistent bloodlessness in general.

As has already been stated the etherized cats of the present investigation were placed on the back or side in postures naturally assumed and with the limbs free.   Some regions were necessarily higher than others and in these regions,—over mid abdomen and chest, with the animal on its back, or over the upper side and flank, when it was on its side,—the spotting tended to last longest after return of the blood. When the cat was in the dorsal posture and the hind legs lay symmetrically half flexed with one knee slightly higher than the other, the ischemia persisted longer over this higher knee even though the difference in level was but two or three centimeters.   The important influence of local pressure differences, as thus illustrated, was especially well to be seen when one of the legs had been raised for some time.

Experiment 7.—The cat was that of Exp. 3 (q. v.).   It lay on the left side, but slightly toward the dorsum.   In order to relieve the abdomen and chest from any pressure of the legs that were uppermost,—those of the right side,—a string had been looped around one toe-nail of each and tied to a support in such wise that the limbs were held suspended in the air in the same posture of partial flexion as their fellows.   The suspended right foreleg must be dismissed from consideration since it had not been epilated.   When the phenol red was injected, after the bleedings, as already described, no stain whatever entered the tissues of the raised hind leg for some little while, but by the time its fellow on the other side had become diffusely orange a well defined, horizontal color demarcation had developed in it just distal to the groin, approximately 5 cm. above the well stained thigh. Above this level the tissues of the leg were pallid and unstained, below it orange.   The zone of transition was about 0.6 cm. broad.   The femoral vein of the raised leg was noted to be collapsed, that of its fellow distended.   Eleven minutes after the return of the blood, when the general body surface was a brilliant pink with scattered orange spots, there was still not the least staining of the suspended leg.   The blood pressure had been far above its initial height since the re-injection of the blood.   After a further six minutes the line of demarcation had disappeared and phthalein was extending upwards into the skin of the leg, staining it orange (or *apricot buff* in Ridgway's nomenclature).   The animal was by now *old*

rose with scattered *apricot buff* patches. Thirty-eight minutes after return of the blood the highest part of the raised leg, the region around the knee, was still only faintly stained (pale buff). This region was 10 cm. above the midline, as the animal lay, but only 3–4 cm. above the highest part of the side. In this part the color had a slight admixture of yellow as compared with the diffuse *old rose* seen elsewhere. The wide-spread spotting had disappeared. The blood pressure had fallen somewhat (from 152 mm. to 134 mm.) but was still above the initial level (120 mm.). The animal had now been under ether for more than two hours.

Experiment 8.—The cat of Exp. 1 (q. v.) served for the observations. It lay on the back under light anesthesia with the legs symmetrically rotated outwards and half flexed. They never became flaccid and hence did not touch the table at all. The right knee was slightly higher than the left. After the injection of the phenol red the tissue for a wide space about the knee was observed not to stain at all, and it remained unstained when the blood had been returned to the body, although the carotid pressure was raised thereby to a much higher level than it had been originally (160 mm. as compared with 142 mm.) and there occurred a wide-spread staining elsewhere. Ten minutes after the injection the superficial tissue about the knees was still uncolored and one could perceive after oiling the skin that this held true of the underlying muscle as well. Yet the main artery of the limb was beating far more violently than usual, owing to the induced plethora, and the pads were red. After another fifteen minutes traces of dye were entering the ischemic tissue, rendering it buff at some places. Elsewhere in the leg one could perceive through the oiled, unstained skin that the large subcutaneous veins were darkly red, with a pink diffusion of dye immediately next them. The animal was in general ruddy, but spotted irregularly with pale orange buff. The blood pressure had remained high. Both legs had attained the same ruddy hue after 44 minutes in all. There was still much spotting over the chest and abdomen. 40 cc. of warmed Ringer's solution was now injected to increase the plethora, and thereafter the spotting soon disappeared.

In these instances the slight circulatory difficulty produced by raising the leg a few centimeters above its fellow sufficed, not merely to render it ischemic after the depletion but to ensure the persistence of the ischemia later, when the blood bulk had been restored and the blood pressure had been brought so high that the circulatory difficulty just mentioned should have been wholly negligible. In Exp. 8 there was an obvious plethora after return of the blood; the main artery of the ischemic limb beat violently, the paw was well stained, and the venous blood deeply so. Yet the tissue previously ischemic remained for some time obdurately bloodless.

Needless to say the factor of local chilling had to be considered as possibly favoring a persistence of the bloodless state, however caused.

The ischemic tissue became cold.   So too, if in a less degree, did the body surface patched with ischemia.   But that the development of such patching is not essentially dependent upon cooling has been sufficiently shown by immersing animals in oil at body temperature (12).   The cats in which its persistence was studied were often long on the table, and care had to be taken to keep them warm in order to prevent shock.

The question whether the spots recur at the same places after a second bleeding is one of great importance for an understanding of them.   Many experiments were done upon this theme, a principal difficulty being the long interval required for complete disappearance of the local ischemia after the blood of a first depletion had been returned to the body.   It was necessary to recognize and rule from consideration the patching due to local pressure differences (of the sort illustrated by Exps. 7 and 8); for such patching infallibly recurred unless the position of the animal had been changed.   Needless to say this recognition and ruling out could not always be perfectly done.   Furthermore there were spurious patchings to be discriminated, as where underlying fatty masses made the skin appear pale, or where the circulation was naturally poor, as about the umbilicus.   In regions of local inflammation the staining was always far better than elsewhere; but such regions were always readily discriminated.   Abrasions with the razor give rise to a staining that is on the actual surface, not beneath it as in the case of the cutaneous staining proper.

The general plan was to use phenol red to disclose the patchings of a first depletion, which were then marked out here and there with dots of india ink; and after restoration of the blood and a second bleeding, —sometimes indeed after return of the blood for the second time,— brom phenol blue was injected.   This dye diffuses swiftly and is more easily seen in the tissues than is phenol red; and hence the pallid patches demonstrated with its aid often appear smaller than when the latter dye has been used—the more especially since this turns orange in acidotic tissues.   The animals were usually bled to death from the carotids while the ischemic patching of the second depletion was still evident; and the pelt was at once stripped to determine the relation of local features to the patching.

Experiment 9.—The cat of Exp. 1 (and Exp. 8) was kept warm and under light ether until all the patching of the first depletion had disappeared. As the protocol of Exp. 8 shows, the process seemed to be hastened by the increase in plethora brought about through the rapid intravenous injection of 40 cc. of warmed Ringer's solution $54\frac{1}{2}$ minutes after the last blood injection. However this may be, a second series of bleeding was begun 141 minutes after the start of the first. The outlines of fourteen of the characteristic patches induced by the first depletion had been marked on the skin with small dots of india ink. The body surface was diffusely stained *old rose* at the time when the new bleedings were begun. The carotid pressure was 144 mg. Hg, the initial pressure having been 142. Hemorrhages of 43 cc. in 19 minutes now caused it to fall to 78 mm. Within 15 minutes after the initial hemorrhage (of 33 cc. in 4 minutes) the phthalein in the tissues turned toward orange where some of the marked spots had been; and 3 minutes after the second bleeding (of 10 cc. in $1\frac{1}{2}$ minutes) the spots had become accentuated and numerous others were present in unmarked regions. The general color was between *light jasper red* and *alizarin pink*. The body surface was warm. The pressure rapidly recovered to 90 but fell to 82 when a further 3 cc. of blood was taken, $5\frac{1}{2}$ minutes after the second bleeding; and it had not risen 2 minutes later when 6.6 cc. of brom phenol blue was injected in 50 seconds. This raised the pressure to 94 but it had again fallen to 78 when, after 8 minutes more, the entire quantity of defibrinated blood, less 2 cc., was reinjected in the course of 95 seconds. The pressure rose from 76 to 140 mm. in the course of 4 minutes. The general condition of the animal was excellent.

During the interval after the injection of the brom phenol blue and prior to return of the blood a patchy staining of the superficial tissues occurred, superimposed upon that with phenol red. There were numerous scattered splotches into which the new stain did not enter, and the plum color of the regions between was not diffuse but dappled with small spots of dirty yellow which the brom phenol blue had failed to penetrate. Following return of the blood these ischemic yellow splotches stood out pronouncedly, owing to an increased staining of the tissue between them. A count made $3\frac{1}{2}$ minutes after the injection of the second stain showed that 8 of the 14 areas previously marked with ink dots were present as such splotches, but of these 8 two were near the projection of the ensiform cartilage and might have owed their freedom from the blue phthalein to local pressure differences. Five marked areas had stained somewhat, and one was not to be discriminated from its surroundings, being deeply colored. The spots that recurred were not accurately reproduced in the original form, and were in general somewhat smaller. The body surface elsewhere was abundantly spotted. Yet the recurrences within inked outlines were too definitely related to these latter to have been due to chance.

Twenty minutes after the blue phthalein had been given the animal was killed by cutting the carotids. The blood pressure at the time was 128 mm., and the ischemic spots had persisted. At autopsy no local factors to account for them could be found. Numerous others were visible in the furred skin when this was stripped back.

These findings, representative of several experiments, leave no doubt that some of the ischemic patches recur in the same situations after the second depletion, though this is far from being true of all. The injection of brom phenol blue had not been necessary to demonstrate the fact in the instance just given in detail. For the animal was still stained with phenol red after the blood was withdrawn for the second time; and patchy changes toward orange, the color indicative of local acidosis, evidenced a recurrence of the local ischemia in the previous situations.

The circulatory readjustment following the first return of blood was in some cases completed only after the animal had been under ether for several hours altogether. In these cases a singular patterning with brom phenol blue resulted, one wholly different from any observed after depletion under ordinary circumstances, no matter how severe this had been or to how low a level the blood pressure had been reduced.

Experiment 10.—The cat of Exp. 2 still showed some spotting 55 minutes after the first reinjection of blood, and 126 minutes after the initial bleeding. Only after another 38 minutes had the staining become diffuse, the color being then slightly darker than *old rose*. The blood pressure was 166 as compared with 140–145 at the beginning. Now, 206 minutes after the initial etherization, 61 cc. of blood was taken, at three bleedings in the course of 57 minutes, reducing the blood pressure to 72. Twelve minutes later the surface was mottled with buff spots on an irregular ground of *coral pink* and *old rose*. 6.6 cc. of 4 per cent brom phenol blue was injected in 75 seconds, and 5 minutes later all the blood was returned to the body, in the course of 180 seconds. The animal began to stain promptly, a plum blue reticulum enclosing greenish yellow patches averaging about 1 cm. in diameter. A count made 9 minutes after the dye had been given showed that only 6 out of 13 areas ischemic previously, and marked as such with india ink, were unstained by the blue phthalein, the other 7 being as deeply, if irregularly, colored as the tissue anywhere else. One could not be certain that the sparing of even these 6 was due to anything but chance, since ischemic patches were everywhere very numerous. Later the 6 largely lost shape through an irregular encroachment of the plum color. Twenty-eight minutes after the injection of the blue phthalein, and 262 minutes from the time of the initial bleeding the blood pressure was as high as at first (140 mm.). The superficial staining had now assumed a regular pattern, thick purple meshes separating rounded areas of dirty greenish yellow about 1 cm. in diameter. This fish-net mottling persisted practically unchanged throughout the next 40 minutes during which the carotid pressure declined to 130. In the course of the next hundred minutes the mottling largely disappeared, but

not wholly. The experimentation had lasted more than 7 hours. At its end the blood pressure was only 95, the rectal temperature 98.6

The disappearance of the reticulum took place by an entry of the blue dye here and there into the greenish yellow spots, breaking them up into irregular areas 3–4 mm. across. Thus the body surface became thickly strewn with little ischemic patches. These very gradually acquired stain and disappeared.

This experiment, one of several with similar results, yielded evidence of a change in the character of the blood service to the superficial tissue of animals long on the table. But its principal use in the present connection is to illustrate a difficulty met in determining whether local ischemia tends to recur in the same situations.

DISCUSSION

The original aim of the work here reported was to learn whether the ischemic spotting which develops after depletion affects the same regions when depletion is induced once again. Our evidence for the obduracy of the ischemia is the more convincing because unexpected. In the literature one finds few signs to suggest that such a phenomenon exists. The methods have not been fitted to disclose it. Yet bedside practice from immemorial times has allowed for it as a possibility. The old wife customs of chafing bloodless extremities, and applying heat to them, are still in current use to "restore the circulation," on the assumption that restoration might long be delayed without such help.

Zak (13) has described a blanching of the fingers when they are exercised after the circulation to them has been cut off. He believed this due to an active contraction of the small vessels, but Rehberg and Carrier (14) hold that the blood is merely forced from the fingers mechanically. These authors do not deal with Zak's further observation that the vessels contract in a frog muscle exercised while the circulation is stopped, failing for some time to admit blood again when the impediment is removed. Whatever the explanation of this latter phenomenon the circumstances of its occurrence are essentially different from those of the one we have studied. Not so however with the patchy areas of ischemia of the human skin known by the name of Bier's spots (15). These areas of local contraction of the small vessels develop gradually when the circulation to a congested limb is cut off

by means of a pressure cuff; and they are evident as blanchings in the midst of a venous hyperemia. In our animals the local cessation of blood flow was induced by depletion, not by a cuff, and the regions of ischemia were demonstrated with a diffusible stain. The numerous reasons for identifying the ischemic patches of our animals with Bier's spots will be detailed in a subsequent paper. Lewis (16) states that the best-developed spots tend to resist the sudden influx of arterial blood after release of the cuff, which floods the arm with the bright pink of active hyperemia. In our own experience with many subjects and numerous trials this resistance has never lasted long. Most of the blanched spots disappeared at once in the flush, and those which did not faded rapidly from the periphery in the course of a minute or two. But it has seemed unwise to cut off the circulation from the arm for more than 45 minutes. The short period of occlusion when taken in connection with the relatively slow metabolic activities in man as compared with the cat, may explain the differing persistences of the ischemia.

Bier (17) pointed out that the pallor of the dead body is due to a generalized contraction of the small vessels, similar to that manifested locally on the living subject in the blanched spots now called after him; and Lewis holds that the dead whiteness of the fingers in Reynaud's disease is referable to the same cause. Our observations bear out these assumptions, and give ground for a further one, namely that much of the difficulty in restoring the circulation to parts temporarily deprived of blood, as in Reynaud's disease, chilblains and skin threatened with bed-sores, comes from a vascular perversion secondary to the initial ischemia. Whether this perversion occurs in those instances of shock and hemorrhage which continue to show a pallid, cold skin despite transfusion and other active measures remains to be seen. The cause for the phenomenon here reported will be discussed in a later publication. It is scarcely necessary to remark that the length of time during which the ischemia lasted in our experiments did not nearly approach that required to cause frank injury of the tissues. Furthermore the deprivation was the result, not of arbitrary interference with the circulation, but of compensatory readjustments within the organism such as would tend to prolong life.

SUMMARY

In skin regions which have been bloodless for some time, as result of the functional readjustments following upon a reduction of the blood bulk, the ischemia persists long after the blood volume has been restored and the systemic blood pressure has mounted to the initial level or a higher one.

The significance of the finding is briefly discussed.

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THE FINAL RESPONSE OF THE SMALL CUTANEOUS VESSELS

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PLATES 24 TO 28

In several previous reports a patchy cutaneous ischemia has been described which develops in animals some time after the blood volume has been markedly reduced (1). Our general procedure to demonstrate it in animals has been to inject a highly diffusible vital dye intravenously after reducing the blood volume by hemorrhage or by anhydremia. Under such circumstances the skin becomes spotted with pallid areas into which the dye has not entered, on an intensely stained ground. The animal in consequence appears piebald. To test for such a state of affairs in human beings one might conceivably compress the main artery to a limb sufficiently to reduce greatly the blood flow to it, and then inject a vital dye into the slight stream still passing the obstruction. Fortunately, there is on record in the literature a phenomenon elicited in man, and still unexplained after thirty years, which nearly parallels both in conditions and consequences our hypothetical experiment. We refer to "Bier's spots." In the present paper the conditions under which these spots arise, their relation to the patchy ischemia of animals, and the significance of both will be discussed.

Bier reported (2) that when the human arm is suffused with blood by obstructing the venous outflow, and all circulation stopped with a tourniquet, a secondary readjustment of the contents of the engorged cutaneous vessels occurs. Pallid spots gradually appear here and there on the violet skin, rapidly increasing in number and size. The blood has in some way been driven out of them. Bier believed that the cutaneous vessels had contracted as result of the stimulus of venous blood, an explanation since voided. The spots have been studied repeatedly in late years but their significance remains uncertain. Rehberg and Carrier (3) discriminated two kinds of patching on the congested arm, the one

pink or light red, and due, so they thought, to the retention of tonus by vessels receiving a little arterial blood through bone-marrow collaterals. Wolf (4) has confirmed the explanation experimentally. The other patches, of blanching, Rehberg and Carrier attributed to capillary contraction as the arm cooled. Wolf has since demonstrated that increasing the local circulation beforehand by heat or friction tends to prevent the spotting; whereas decreasing it, by cold, causes more of the spots to appear. She pointed out that the known factors conditioning the spotting, (a) state of the local circulation prior to the occlusion and (b) the temperature of the skin during it, cannot be the sole influences determining the spots since the utilization of them does not enable one to blanch large areas of skin at will. Recently Lewis (5) has decisively excluded cold as the primary cause for the blanching. He holds it due to vaso-constrictor substances developing in the tissues.

Likenesses of the Spots and the Patching

As the foregoing summary discloses, the conditions which lead to Bier's spots differ in certain respects from those which result in the ischemic patching in animals. In the one case the circulation is completely stopped, whereas in the other it is reduced greatly through compensatory vascular readjustments. In the one the small cutaneous vessels are primarily engorged, and contraction of them becomes evident as it empties them of blood. In the other these vessels are largely empty to begin with and the contracted state is disclosed by the patchy distribution of a vital stain. We have made repeated attempts to elicit Bier's spots in white rabbits, cats and a white pig, but with dubious success. On venous occlusion the skin does not become engorged as in man, and the spotting seen after arterial closure is ill-defined. Hence we have resorted to the intensive study of Bier's spots in human beings, hoping to learn whether the conditions which differentiate them from the ischemic patching are essential or accidental.

Bier's spots are readily produced in most individuals, but best in persons with a fair skin not greatly freckled or tanned. In some the spotting develops pronouncedly, whereas in others it is slow to appear and the blanching is incomplete. Our subjects were volunteers.

In appearance the spots resemble the ischemic patches in stained animals. Both are scattered, of highly various size, small at first and growing by peripheral extension and coalescence into large areas with more or less sharp-cut, serpiginous margins. In animals stained

beforehand with phenol red the development of ischemic patching can be followed by means of the color change attendant upon the resulting tissue acidosis. The bloodlessness of the regions affected was proven by their failure to stain with a second highly diffusible dye injected intravenously. Vital staining was done only after the depletion. Using these methods the progress of the ischemia was studied in a series of animals. It first appeared some minutes after the local circulation had been cut down by hemorrhage and the longer the interval the more widespread and pronounced it was. Many of the spots, though lying in a tissue still fairly served with blood as shown by the staining, were completely ischemic, none of the dye penetrating into them during long periods. The facts are similar for Bier's spots. An interval elapses before they appear, and they gradually become numerous, extensive and more completely blanched.

The Influence of Vascular Distension

Passing now to points of difference, one encounters first the fact that Bier's spots develop in a skin suffused with venous blood, whereas the ischemic patching appears in one already notably anemic. Our first task has been to determine whether the suffusion is a necessary preliminary to the spotting or merely renders it visible. A lead had been provided through the observation that in animals the ischemic patching, though scattered broadcast over the body, tends to be pronounced wherever local pressure differences would act to empty the vessels (6). For example, it is especially marked over the backbone and knees of rabbits crouching naturally, and over the uppermost portions of the prone body. The patching at such situations is distinguishable from that elsewhere only in its shape, as determined by local factors, and in the evident influence of slight differences in tension, or pressure, to determine the localization. We have asked ourselves whether such differences can act to determine the situation of Bier's spotting. That this is the case can readily be shown.

Test 1.—The pressure cuff of a Tycos sphygmomanometer was placed high on the upper arm of a young man (R.), and his blood pressure was taken (102 mm. systolic, 80 mm. diastolic). The subject was seated beside a table with the arm in a natural position over a large mirror which it was kept from touching by a support beneath the cuff and another at the lower edge of the palm. The lower arm

was horizontal with its radial side highest.   The pressure in the cuff was brought to 70 mm. for 5 minutes and then abruptly raised to 180 mm.   Within 10½ minutes from the beginning of the experiment Bier's spots had begun to appear upon the greatly congested skin of the arm and hand, and after 18½ minutes they were many, large, and greatly blanched.   Nearly all were on the upper surface though some few extended as far down as the palmaris longus; and in the mirror a single ill-defined one was visible on the under side of the arm.   The thenar eminence showed numerous pallid splotches.   The pressure in the cuff was now released.

After an interval of a half hour to allow for the return of normal conditions the experiment was repeated with the subject in precisely the same position save that the lower arm was rotated inwards so that the thenar eminence and radial prominence were on the under side.   After 13½ minutes of complete occlusion of the vessels the spotting was as pronounced as before, and again it affected only that part of the arm and hand which was uppermost, precisely the part that had previously been free from it.   The thenar eminence, which had become so brilliantly blotched after the first occlusion, showed no spotting whatever nor did that portion of the arm which had formerly been uppermost and was now on the under side (Fig. 1).

Another half hour was permitted to elapse, and then the test was repeated in the position first assumed.   The findings were identical with the initial ones (Fig. 2).

In some subsidiary observations made two days later the subject of these tests lay prone on his right side with the left arm stretched horizontally across the mirror, and supported above it as before.   The limb was so rotated at the shoulder that the ulnar side of the forearm was highest throughout its extent as had not been the case previously.   The many Bier's spots developing in 14 minutes of vascular occlusion were all in the skin of this upper, ulnar side.

In experiments like this one can perceive, in subjects with a thin, fair skin, that during the preliminary venous obstruction the blood tends to accumulate on the dependent side of the arm, only gradually mounting into the skin that is highest.   Even when the preliminary period has lasted seven minutes, the upper side of the limb may still be paler and pinker than the lower.   After arterial occlusion the difference in hue is soon lost, both sides becoming violet blue; but the difference in shade continues and is accentuated, not merely by the appearance of Bier's spots on the upper side, but by a gravitation of blood from it into the dependent regions, which become more intensely suffused.   When the cuff is relaxed bright blood leaps at once into the empty vessels of the skin of the upper side, a brilliant pink hyperemia appearing there.   Further down the change lags, and

often one can see, cloudlike masses of dark blood gradually moved, broken up and dispersed in the sweep of bright blood.   The entering stream has to displace little or nothing from the cutaneous vessels of the upper side of the arm but much stagnant blood from the lower.

It has been our general habit since Test 1 to study Bier's spots on the propped arm.   The position hastens and localizes the spotting. Regularly Bier's spots appear earliest, and are most abundant and whitest, in the skin of the upper side.   (Figs. 2, 10, 14, and 15.) Nearly always they are absent from the under side, even when the arterial occlusion has lasted forty five minutes; and usually none have developed more than half way down.

These facts escaped the attention of previous investigators for reasons that become evident when one reads their protocols.   The arm with occluded vessels has either been let hang vertically, the local differences in spotting being attributed to anatomical differences in the skin of forearm, palm and fingers,—or it has been rested directly upon a table with result that blood could not accumulate in the skin of the under side, and that this skin could not be seen.   The position of the propped forearm which would seem to be most favorable to Bier's spotting is that of horizontal half extension, thumb up.   The boneless lower side of the arm into which the blood drains is flaccid under such circumstances, and the spots appear first along the ridge over the radius, in a region that is to say that is emptied not by gravity alone but by some actual pull on the skin over the bone by the heavy, sagging parts.

Although the data thus far presented give good reason for the supposition that suffusion of the vessels acts to hinder rather than to induce Bier's spotting this cannot be concluded forthwith.   For the upper side of the arm, gradually emptying of blood, would tend to grow cool sooner than the lower, and cooling favors the spotting.* In the test which follows, the under side of the arm was chilled in water at the temperature optimal for spotting (7).

Test 2.—The same subject (R.) was employed as in Test 1.   Preliminary observations were carried out to determine whether the portion of the skin destined to be chilled later would develop spots if the arm were so rotated as to bring it

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* We were baulked at first in attempts to elicit blanching under the Cooper Hewitt light for purposes of photography, by the heat that these lights threw out; but when the room temperature was reduced to 70° by a draft of air the difficulty was no longer encountered.

uppermost.   For the purpose R. lay on his left side upon a table, with his right arm fully extended and horizontal at the shoulder level.   The forearm was propped as usual above a mirror.   The olecranon and ulnar border of the hand were the highest points.   The pressure in the cuff on the upper arm was raised to 70° for 4 minutes and then kept at 170 mm. for a further 27 minutes.   During this period numerous Bier's spots developed on the upper surface of the arm and hand, a few on the sides, but none below.

An hour after release of the pressure cuff the subject was placed at a table, the right forearm horizontal and propped as before, but this time with thumb and radial side up.   After 4 minutes of venous engorgement the artery was occluded with 170 mm. pressure, and the arm was lifted by an assistant and placed in a large fish-kettle nearly full of water at 6½°C.   Only the lower half of the little finger and the skin of the under side of the hypothenar eminence and forearm were submerged.   A single prop sufficed to hold the limb in place, one under the wrist. On the bottom of the kettle was a flat dish full of clean mercury which served as a mirror.   The temperature of the room was 20°C., as in the preliminary observations.   After 11 minutes of arterial occlusion numerous Bier's spots had developed on the upper side of the forearm, and several on the dorsum of the hand 1½ minutes later.   There were some also on the sides of the limb, extending in diminishing number and intensity almost as far down as the water line.   After 28 minutes there was still not a single one to be seen in the submerged cutaneous tissue, though now the whole upper side of the forefinger was blanched and there were many large spots on the upper side of the hand and arm.   Those situated on the sides had increased but little in size and not in number.   The temperature of the water had risen to 8½°C.   The arm was now suddenly lifted out and the portion previously submerged was inspected directly for spots.   There were none.   The cuff was released.

The results of this test, and of one like it on another subject, rule out cooling as the effective cause for the localization of the spots to the upper side of the propped arm.   Tests were next undertaken to learn whether the spotting would be more abundant if the cutaneous vessels were emptied artificially instead of by gravity.

Test 3.—The subject K., a healthy young man, systolic blood pressure 110 mm. Hg., diastolic 80 mm., was seated at a table with the arm propped above a mirror, thumb up.   The arm was long, slim, thin skinned, fair, with but scanty hair, prominent veins, and almost no skin markings.   The pressure in the cuff placed over the biceps region was raised to 70 mm. and three minutes later to 170 mm. at which it was maintained.   By this time the numerous large veins had become greatly distended and the skin was everywhere violet.   Now as rapidly as possible an Esmarch bandage was wound tightly about the thumb and each finger, beginning with the tip, and it was continued over the hand and up the arm to a point

nearly half way to the elbow. The bandaging was done rather slowly, both because it proved painful owing to the great engorgement, and to allow time for the blood forced out of the tissue to be accommodated in the upper arm. Immediately above the Esmarch a tourniquet was laid on to prevent return of the blood to its original position. This tourniquet consisted of strong rubber tubing which flattened out into a strap 2 cm. broad when wound about the arm. The bandage was then removed. The entire process had taken four minutes. It could now be seen that all the region below the tourniquet was pallid save here and there where the pressure exerted by the bandage had been uneven and a little blood had been caught between its folds, remaining behind as a pale violet blotch. The hand looked like a dead hand. On the portion of the arm above the tourniquet the skin was dusky violet, and a few fresh punctate ecchymoses could be discerned. Seven minutes later it was evident that a considerable quantity of dark blood had passed the barrier of the tourniquet from above, owing to the poor situation of the latter on the thin, bony arm. This returned blood was not distributed evenly, nor was its situation haphazard as had been the case with the little left after the bandaging. It lay in well defined patches suffusing the skin between very numerous spots, characteristic Bier's spots. One of the largest blanched areas covered a part of the under surface of the wrist. There was at this time no spotting whatever on the congested arm above the tourniquet, but a few more ecchymoses had appeared there. Three minutes later the condition was essentially unchanged (Fig. 3). Within another three, however, Bier's spots had developed in the greatly congested skin above the tourniquet. They were small and incompletely blanched, the largest only 6–7 mm. in diameter. Two were on the lower side of the arm. The violet congestion had no greater intensity on this side than on the upper one, for the obvious reason that the vessels were everywhere filled to a maximum. At the time the tourniquet was taken off, after 9 minutes in place, the contrast between the engorged and emptied segments of the arm was extraordinary (Fig. 4). Below the tourniquet there was a leopard mottling, numerous brilliant, white blotches, many of them sharp edged, being scattered everywhere save on the under surface of the wrist. Here and on the dependent thenar eminence some less well defined ones could be made out. Many of the others that were most blanched lay between, not upon, the bony projections of the hand, though in general they were localized in the highest skin regions. The cutaneous tissue between them appeared suffused with blood. The engorgement above the tourniquet was no greater on the dependent side of the arm than on the upper one,—the violet color being the same in both situations; and the few small spots of relative pallor had not progressed in either size or intensity. They cannot be made out in the photograph. No pink spots were visible anywhere at any time. Just before the tourniquet was removed the arm was changed from the horizontal position by lowering the prop beneath the hypothenar eminence 4.5 cm. This was done that gravity might aid in the distribution of the venous blood later. Immediately after the loosening of the tourniquet there was a rush of dark blood into the lower

arm and hand, with an instantaneous partial obliteration of many of the spots previously present. Yet the most pronounced of them remained largely uninvaded by blood during the succeeding three minutes before the pressure cuff was relaxed (Fig. 5). During this period no new Bier's spots developed in the skin of forearm and upper arm, which had been suddenly relieved of its special engorgement; and the few small spots already present did not enlarge or become more distinct.* With the artery freed all previous differences were submerged in the sweep of an active hyperemia. When this had subsided it could be seen that the skin everywhere in the segment that had lain between cuff and tourniquet was evenly speckled with ecchymoses. Below the situation of the tourniquet none were found.

In this test the blood was forced from part of a congested arm into another part which in consequence became so engorged that some of its small vessels ruptured. But even in the extremely engorged tissue Bier's spots appeared. They were as nothing, though, compared with the profusion of great spots over the emptied forearm and hand. The partial obliteration of many of these spots when the venous blood was allowed to return to its original situation proved due, as later tests showed, to the unusually slight tendency of the subject to Bier's spots. Almost none appeared on his arm and hand when they were subjected to ordinary congestion and occlusion. In another individual, with skin reacting more characteristically, the spotting resisted the reflux of venous blood and was accentuated thereafter.

Test 4.—This was carried out like Test 3 but on a middle-aged man (P.R.), whose arm was shorter and thicker, thin skinned but with many freckles. His systolic pressure was 120 mm. Hg, diastolic 80 mm. The artery was occluded after only $2\frac{1}{4}$ minutes of venous congestion. Four minutes was required to force the blood out of hand and lower arm with the Esmarch bandage, and apply the tourniquet. There still remained an irregular pale violet tint here and there on the wrist and the back of the hand, one so slight that it is not evident in the photograph (Fig. 6). The hand now looked like that of a cadaver. During the succeeding 16 minutes with the tourniquet in place some small, pale patches developed in the skin above it, turgid though this was with the excess blood forced in. The patches were not localized to the upper side of the arm. Below the tourniquet the surface veins gradually filled, though under very slight pressure, and sufficient blood reentered the skin to demonstrate the existence in it of extensive regions of vascular contraction, regions demarcated as such by the failure of the blood to pass into them. These regions were larger and more numerous on the upper side, but there

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* For the purposes of photographing Fig. 5 the arm was raised temporarily to the horizontal.

were some in the dependent regions and others that extended into them from above. Especially notable was a blanched area several centimeters in diameter about the ulnar prominence. The entire radial side (upper side) of the forearm was covered by a huge, bloodless spot extending around irregularly to the lower surface of the wrist. Over the region about the knuckle of the index finger was another, less extensive blanching.

Now the tourniquet was released. Just previously the hand had been lowered 2.2 cm. in order to facilitate the return of blood to it, and a little afterwards it was lowered 2.2 cm. more. The height of the prop under the upper arm was not changed. The blood rapidly found its way back, even to the tips of the fingers, and its presence threw into relief on a violet ground the extensive pallid areas (Fig. 7). In the six minutes that elapsed before the circulation was again allowed to enter the limb, these areas became sharper-outlined and progressively more blanched, to a dead white. The one which lay over the radius now extended rapidly into the skin where the tourniquet had pressed, through tissue that still bore the deep marks of it, and into that portion of the arm which had been forcibly congested (Fig. 8). The few small spots previously present in this latter became more clearly marked, but were, to the end, far smaller than those developing in the same region of the same subject under the ordinary conditions whereby Bier's spots are elicited.

When the cuff was relaxed all of the blanched patches were lost at once in the active hyperemia flooding the skin, except that about the base of the first finger, which very gradually faded. In the region above the tourniquet, which had been so greatly congested, the skin was evenly stippled with minute ecchymoses. These were no more numerous where the little blanchings had been than they were elsewhere.

At no time after the Esmarch bandaging were any arterial spots, "red spots," visible, though some had been seen about the wrist during the preliminary period of venous engorgement.

These findings confirm Test 3 and, like it, afford conclusive evidence that the emptier the vessels are, the greater is the tendency to that secondary contraction of them which produces Bier's spots. The other arm of the subject P. R. was submitted to the same test, though with a preliminary period of engorgement lasting 3 minutes; and essentially the same phenomena were observed. Once again an enormous blanched patch developed over the radius, extending from tourniquet to wrist, and rapidly becoming dead white and more sharply demarcated after the reflux of venous blood. Under the ordinary circumstances which lead to Bier's spotting, and after the same time at the same temperature, the subject showed no spots more than 2½ cm. in diameter.

Test 5.—In some subsidiary tests on P.R. the arm was largely emptied of blood before pressure was thrown into the cuff, by raising it above the head and contracting its muscles. Then a pressure of 180 mm. of Hg was abruptly thrown into the cuff and the limb was lowered and propped in the usual horizontal position above a mirror. It was pallid, with but slight distension of the surface veins. After some minutes the dependent side of it became pale violet because of an accumulating small quantity of blood, and here as time went on characteristic Bier's spots were to be seen. All over the upper, radial, side of the arm, which was pale as before, one could make out dimly a great saddle-shaped blanching with ragged edges demarcated by the hyperemia below. After the lapse of nearly half an hour the pressure in the cuff was lessened, till at each inspiration a little arterial blood passed its barrier. This first appeared as a pink flush on the under side of the arm, which, gradually mounting, obliterated some spots and placed others in sharp relief. With due care taken that the influx of blood should be gradual, the last mentioned spots persisted although they were surrounded and demarcated by bright arterial blood. After the arm had become fairly engorged the circulation was once again cut off. Within two minutes many Bier's spots that were entirely new sprang into view, sharp edged and strikingly pale, replacing here and there a pink hyperemia which had only just begun to take on a violet cast.

These various facts prove that suffusion of the vessels is not necessary for Bier's spotting but merely renders it evident to the eye. They show that one of the main differences between the conditions associated with the development of Bier's spots and the ischemic patching in animals, namely fullness and emptiness, respectively, of the vessels, is superficial in nature. Instead of favoring Bier's spotting distension of the vessels acts to hinder it. The spots develop much more pronouncedly when blood channels are almost completely emptied, that is to say when they are in the state that one finds associated with the ischemic patching of animals.

The Influence of the Character of the Blood

The patching develops while there is still a dribble of blood through the tissues, whereas Bier's spots, as ordinarily elicited, appear in tissue from which the circulation has been wholly cut off. Will they develop, like the patching, in skin still supplied with a little arterial blood? It is easy to show that this is the case.

Mention has been made of the "red spots" fed by arterial collaterals which appear about the hand and wrist after occlusion of the

vessels in the upper arm.* Bier's spots can be observed to develop secondarily in the midst of not a few of these. The fact is demonstrable either by following the late changes in "red spots" outlined with dots of india ink, or by inducing relaxation of the vessels of Bier's spots, noting the color of the blood that flows into them and the happenings thereafter. Lewis found that stroking the spots causes them to disappear (8). We have employed either this or gentle tapping to induce relaxation. The color of the blood that surges in discloses whether it is arterial or venous. And there is another, subsidiary criterion. Rehberg and Carrier noted that the blood entering "red spots" blanched by pressure direct wells up from underneath, whereas after pressure elsewhere on the congested arm it rushes in from the violet tissue round about. When the vessels of Bier's spots are abruptly relaxed the direction from which the blood comes is indicative of its derivation.

* The "red spots" appear pale by contrast with the tissue about them. Rehberg and Carrier suppose the tone of their vessels to be maintained by the arterial blood, the result being that they do not become engorged like those about them. Our findings support this explanation, as do those of Lewis on the pallor existing about telangiectases. In five healthy individuals out of eight serving for the study of Bier's spots we have observed local blanchings of special character, well marked, pale areas surrounding little vascular naevi on the congested arm. They came to attention late in a long preliminary period of venous engorgement, and persisted for the first ten or fifteen minutes of arterial occlusion, sometimes longer. So small was the central naevus often that it not infrequently escaped notice before the arm was engorged and then appeared as only a minute point. Even in such instances the surrounding zone of pallor had often a radius of more than one half centimeter.

The blanchings about naevi, instead of intensifying like Bier's spots, tend to disappear as the period of stasis lengthens. That they are attributable,—like the pallor of the "red spots,"—merely to a local persistence of vascular tone in areas unusually well supplied with blood because of proximity to the naevi, was indicated by an involuntary experiment. One of us had a slightly raised, discoid, bright red birthmark, about 1½ millimeters across, in the skin of the flexor surface of the forearm. Pronounced blanching could be elicited for approximately one centimeter around it. As result of the frequent demonstration of the phenomenon clotting occurred throughout the naeval vessels. The mark became purple and was no longer to be obliterated by pressure. From the time of these changes the blanching could not be induced, nor can it now when the clot has disappeared without return of the abnormally abundant circulation.

Two protocols out of many will suffice.

Test 6.—Subject P. R. The arm was rested on props as usual, in a position midway between pronation and supination, the bases of the thumb and forefinger being highest, and at the same level. The period of preliminary venous engorgement was $7\frac{1}{2}$ minutes. Here and there amid the increasing violet hue of the arm could be seen pink spots, regions where the arterial supply must have been especially direct. These were outlined with fine dots of india ink. Soon after the artery was occluded most of them turned violet; but as the general color deepened one saw amidst it, after $4\frac{1}{2}$ minutes, several persisting pink spots about the base of the thumb and forefinger. After 7 minutes white blotches were developing on the upper side of the arm and hand, and after 14 minutes they were well marked. Special note was made of their relation to the pink spots. Three of the latter about the thumb and forefinger endured for some time as such, but gradually two of them were replaced in large part by complete blanchings without any intervening period of violet coloration. Within the confines of these two, pallor and pinkness were juxtaposed. The third remained pink to the end of the observations. Bier's spots also developed in, or encroached upon, some of the violet regions marked with ink, where the skin had received arterial blood during the initial period of engorgement.

Test 7.—The subject was a young woman, K., with a systolic pressure of 104, diastolic 75. She sat beside a table with the right arm propped above a mirror as usual, thumb up. The arm was plump, tanned on its extensor surface but with thin white skin on the flexor. Few veins or other natural markings were visible. The preliminary period of venous engorgement with the cuff at 60 mm. Hg pressure lasted 6 minutes. As the arm filled one could perceive pink areas here and there amid the pale violet, in special one about the base of the thumb. Now the pressure was raised to 160. A minute later the arm appeared everywhere of the same light violet save on its upper surface where were a few pale flecks,—beginning spots. After 7 minutes of arterial occlusion these spots were much larger and better defined, situated characteristically on the upper side of the limb, especially near prominences on the hand and over the veins of the forearm. There was a very large and pallid one around the base of the thumb. This was now lightly and repeatedly tapped with the smooth, round head of a small hat-pin. Practically at once it melted away and was replaced from underneath by an area of active hyperemia very vivid against the surrounding violet. Three other spots situated respectively at the base of the thumb, about the knuckle of the forefinger, and on the inside of the wrist were similarly treated, and these were also replaced by pink areas. The four largest spots on the forearm were now tapped. They too disappeared, blood rushing in from the sides to engulf them; but this blood was violet not red, and they were completely obliterated for a little while, gradually reasserting themselves. About the knuckle, metacarpal and thumb the spotting also reappeared, developing in the midst of the induced pink patches, then still of

either a frank pink or violet pink color.   Tapping them a second time caused two of them to be replaced by the same violet hyperemia as existed in the surrounding tissue.   Only the one over the thumb was again replaced by bright blood.   The procedure was repeated a third time and with the same results.   The cuff was released after $15\frac{1}{2}$ minutes of occlusion.   Some of the spots over the thumb and hand remained pallid for many seconds after the rest of the arm had become brightly hyperemic.

The force of the tapping was no greater than proved just sufficient to drive the blood momentarily from normal skin of the same subject.

The method employed in the first of these tests, namely observation without interference, is the preferable one.   For, as will be shown further on, stimulation of the cutaneous vessels during stasis often causes an enduring response of them such as cannot but complicate the situation.   A survey of many instances enables us to state that Bier's spots develop far more frequently in "red spots" than elsewhere on the congested arm.

The amount of arterial blood which passes the cuff by way of collaterals is exceedingly small (9), and there can be but little circulation through the "red spots" which are the sole cutaneous regions to receive it.   Test 7 indicates that when the vessels of these spots close down the arterial blood accumulates behind the obstruction, to flow once again into the tissue when it is removed.   Irrespective of this a certainty emerges from the observations, namely, that Bier's spots will develop in regions with tonically contracted vessels receiving a little arterial blood, that is to say in tissue placed under essentially the same conditions which lead to the ischemic patching of animals. True, the vessels of the "red spots" are not nearly so empty as those of the pallid skin which shows this patching.   Were they as empty one would expect Bier's spots to occur earlier and be still more pronounced (*vide* Tests 3 and 4).

The major differences in the local states leading to the ischemic patching and Bier's spots have now been dealt with.   It is plain that the differences are not basic in nature, but are incidental to the methods of experimentation.

Recurrence and Persistence of the Blanchings

Bier's spots tend to recur in the same situations (10).   One reason for this we have disclosed in the influence of local pressure differences.

If the arm be in exactly the same position and state at each test one would expect that the spotting, as determined by such influences, would be in the same places at successive tests. But if the limb be turned ever so little, or the muscular tensions within it altered there would almost certainly be a shifting. This will explain the failure of some observers to note recurrence (11). Only the crudest, most obvious, of the local pressure factors come to the eye of the observer. He cannot perceive those interior strains, stresses and structural differentiations which must largely determine the situation of the blanchings.

Lewis has called attention to the fact that blanchings frequently occur over distended surface veins and may mark out their course. He has tentatively advanced the explanation that the skin is cooled here more than elsewhere by the blood passing up from the hand, though, as he further remarks, thermopyle observations have failed to disclose such a cooling. In the course of our own studies blanching has very often been noted over distended veins and observed to recur there time after time, but this has been only when the veins were upon or near the upper surface of the arm. When the arm was so rotated that they were brought to the dependent side there was ordinarily no spotting whatever in the skin covering them (Figs. 1 and 2), or at most a faint one. It is clear that the spotting over veins merely constitutes a special case of the influence of local pressure differences. But whether these, as determined by postural and structural anomalies, will account entirely for the distribution of the blanchings is doubtful. Bier's spots do not occur over all the distended veins on the upper side of the arm nor even all along the course of a single one. The blanching is not broadcast on the skin of a hand emptied of blood (Tests 3 and 4) but is discrete and sharply localized. Rehberg and Carrier have advanced the view that the small vessels are more sensitive in some places than in others, those of certain areas contracting promptly to stimuli that are ineffective elsewhere.

In numerous experiments reported separately (12) we have sought to determine whether the ischemic patches of animals recur in the same situations. Such of them as were obviously conditioned by local factors regularly did so, and so also with many of which this was not manifestly the case. Others failed to recur, and new ones appeared in different situations. At the time of the work the importance of precisely duplicating the original posture and conditions was not recognized. Both as concerns recurrences in the same situation and in the failures to recur the patches in animals resemble Bier's spots.

During the experiments referred to, which involved the reinjection of blood, it was repeatedly observed that the ischemic patching persisted for a considerable time after the blood volume had been restored and the systemic blood pressure had mounted to or beyond the previous level. Indeed, with the improvement in the circulation the patches often became more brilliantly demarcated than before. One is reminded of Lewis' observation that Bier's spots stand out more strikingly when the arm is placed in warm water, evidently because of a greater engorgement of the vessels about them. He noted furthermore that under ordinary conditions the most pronounced spots disappear only gradually after the pressure cuff is relaxed despite the bright, general hyperemia (13). In our own experience spots have never persisted for more than a minute or two. Their failure to last as do the patchings of animals when the blood bulk has been restored may conceivably be due to the relatively brief period during which the circulation is cut off from the arm and the slow metabolic rate of man as compared with the rat, rabbit, and cat.

The identification of the ischemic patching in animals with Bier's spots in man would seem in view of all the foregoing to be reasonably complete. But there remains the task of determining their cause.

Proximal Causes of the Contraction

What impels the small vessels to contract? There is much to show that when the circulation through them is cut off they become hyperirritable. Török and Rajka (14) have reported that stroking the arm strongly soon after congestion and arterial occlusion leads to an especially great dilatation of the cutaneous vessels. We have noted, both dilatation and prolonged contraction as result of stimuli ordinarily negligible.

Test 8.—The subject was a young woman, M.T., blonde with a plump arm and fair, thin skin, systolic blood pressure 124 mm. Hg, diastolic 80 mm. The arm was propped above a mirror, thumb up. Almost immediately after the pressure in the cuff had been raised to 70 mm. a white spot appeared on the inner side of the wrist, and with the lens a pin-point naevus could be discriminated at its center. Such had been searched for previously but not found. After 7 minutes of gradual venous engorgement the pressure was raised to 160 mm. Already pallid spots had begun to appear on the upper side of the arm. 9 minutes later, when there was a characteristic spotting in this region but none lower than about two-thirds

the way down the side of the arm, stimulation of the skin was begun. It was repeatedly and gently tapped here and there with the rounded head of a hat-pin. When the tapping was within a centimeter of a Bier's spot this as a rule disappeared promptly, though temporarily, by a flushing with venous blood from its surroundings. Tapping on the dependent side of the arm, where there were no blanchings, caused the skin that was directly struck to become a deeper violet than elsewhere. Well defined, greatly engorged areas about 0.5 cm. in diameter could be thus elicited at will. A row of them were in this way brought into existence low along the inner side of the arm (Fig. 9). About each was a broad areola, or halo, approximately 2 cm. wide, of skin paler than its surroundings. This paling developed at the same time that the central spot did. Both persisted for more than fifteen minutes (Fig. 10), being gradually lost thereafter in the general congestion.

Despite a considerable venous pressure, great engorgement, and long stasis the small vessels most unfavorably situated in this subject, those on the lower side of the arm, though engorging more and more with blood from above, still retained some tone as shown by the increased engorgement when they were stimulated mechanically (Figs. 9 and 10). Blood from the surrounding tissue then flowed into them with result that the vessels yielding it were partially emptied of their contents. They did not fill again to the previous extent but remained for a long period more contracted than those elsewhere, as was plain from the local pallor (Fig. 10).

One can suppose that in this instance the mechanical stimulus caused merely a dilatation of the small vessels directly affected, the active contraction of those adjacent occurring in response to an emptying of them into the central patch of skin. Or perhaps contraction was induced by transmitted tension on the skin, a cause suggested for the pallor peripheral to the reflex flare from stroking (15). The first explanation will not cover the following case in which the lightest touch, one insufficient to displace any blood, caused extraordinary blanchings.

Test 9.—The subject R., of Test 1, was seated with the left arm propped horizontally at the shoulder level, above a mirror and rotated internally, with the thumb hanging down and the palm of the hand resting upon a box. The mirror was slanted so that photographs of the skin on the under side could be obtained. The pressure in the cuff was raised to 70 mm. Hg for 4 minutes and then to 170 mm. Five minutes later Bier's spots had begun to appear over the arm and hand. After 25 minutes of arterial occlusion a photograph was taken to show the distribution of spots on the dorsum of the hand between the forefinger and thumb

(Fig. 11). Immediately thereafter the skin was very gently stroked with the rounded head of a hat-pin in a region where the only spotting was a few, very scattered white dots. The stroking was continued for about 15 seconds, over a circular area about 2 cm. in diameter, and it was so light as not visibly to depress the skin. When carried out on the normal hand of the same subject it caused merely a tickling sensation and no evident vascular reaction. A few seconds after it very white Bier's spots sprang into view in the region stroked, so white and so sharply demarcated as to give the appearance that the skin was raised. In Fig. 12 these spots are shown and the center of the area previously stroked is indicated with the hat-pin. After a minute the stroking was repeated in another purple region and with the same results (Fig. 13). There now remained only one large unblanched region on the dorsum of the hand. This in turn was stroked, and it too paled. A photograph $1\frac{1}{2}$ minutes later shows how widespread was the blanching, none of which had faded (Fig. 14). From time to time the dependent thenar region had been stroked, but without eliciting the least change. No blanching appeared there at any time.

After 33 minutes in all the pressure in the cuff was relaxed. The spots promptly disappeared.

The reflection of the under side of the arm and the thenar eminence in Fig. 14 discloses that they were free from spots. But when, shortly after on the same day, the limb was placed with thenar eminence up, and congestion and occlusion were produced, the regions previously free promptly became spotted, whereas the part of the arm that was now lowest showed no blanchings save for one spot associated with a birthmark (Fig. 15).

In this instance intense blanchings sprang into existence, as result of a pressure insufficient to displace the blood from the vessels.

Wolf elicited Bier's spots on arms paralyzed by nerve severance and watched them spread upon sensitive and insensitive skin alike. To Lewis this is proof positive that nervous activity has nothing to do with them. He invokes as their cause vaso-constrictor substances formed within the skin after the circulation has been cut off, and eventually accumulating in such strength that their influence dominates over a vaso-dilator substance also assumed to be present. To account for the prompt disappearance of the blanchings when blood is again let into the arm the further assumption is made that the constrictor substances are so highly diffusible as to be flushed out and away practically at once while the dilator substance, remaining behind, exerts its characteristic effect. The disappearance of Bier's spots on stroking he attributes to a local redistribution of blood, whereby the tissue is relieved of its excessive concentration of vaso-constrictor substances.

Certain of our data make difficult acceptance of these various suppositions. Bier's spots occurring where "red spots" previously were, can be temporarily flushed with arterial blood from the underlying regions by mechanically stimulating the vessels to relax. But they become red for only a minute or so and then blanch once more (Tests 5 and 6). Surely a vasoconstrictor substance with the diffusibility necessitated by Lewis' conception would have been diluted if not carried away by the influx of arterial blood, and blanching could not have recurred so promptly. In the test which follows, the stroking of one Bier's spot led to the appearance of another separated from the first by a belt of engorged skin. The passive transfer of vaso-constrictor substances out of one cutaneous region into another will scarcely explain such a phenomenon.

Test 10.—The subject D. D. (systolic blood pressure 120 mm. Hg, diastolic 80 mm.) had a long, fairly nourished, thin skinned arm, slightly and evenly pigmented, and an unusually pink hand. The limb was propped over a mirror as usual, with the knuckle of the forefinger as its uppermost point. The pressure in the cuff was raised to 70 mm., and to 160 mm. after 7 minutes. 6½ minutes later the whole lower side of the arm was noted to be much more congested than the upper where already numerous scattered pale patches had developed. A minute later a small, circumscribed pale patch about 1 cm. in greatest diameter, lying over the first metacarpal, was repeatedly tapped with the rounded head of a hatpin. It promptly disappeared, venous blood surging in from the sides, but within less than half a minute a blanched patch suddenly appeared about 1 cm. distant from where the previous one had been and wholly separate from it, over the second metacarpal, that is to say well above the previous spot. This new patch was about 3 cm. in greatest diameter, much more blanched than the original patch, so white and distinct indeed as to suggest urticaria, though none was discernible on palpation. No apparition of the sort occurred elsewhere on the arm at any time, save in response to the stimulus of tapping. This stimulus was applied to several other spots during the period before the cuff was relaxed, after 18 minutes of total occlusion in all. Some of the blanchings disappeared for good, others disappeared and gradually recurred in greatly enlarged form, while in the neighborhood of yet others new and more or less completely separate blanchings sprang into view. Tapping the skin on the upper side of the arm in unblanched regions caused a blanching that proved persistent to appear here and there; but on the under side no response followed. Only on the hand were the Bier's spots numerous or large.

In this and like tests the stimulus which caused relaxation of the vessels of Bier's spots brought about contraction of those in the

neighboring engorged skin. It may well be that throughout the cutaneous tissue a constrictor substance had accumulated which became effectual only when some other stimulus, here a mechanical one, was added to its own. But it will be safest at present to go no further in general explanation of Bier's spots than to suppose that the contractile cells of the small vessels, when no longer properly served by the circulation, become abnormally irritable, responding with contraction (or with dilatation) to stimuli that are ordinarily ineffective.

DISCUSSION

The identification of the ischemic patching in animals with Bier's spots rests upon likenesses which can be summarized as follows:

Ischemic Patches in Animals	*Bier's Spots in Man*
They are blanched areas of skin, similar in appearance and enlarging in the same way. They develop secondarily in tissue no longer well served by the circulation.	
The patches are rendered visible by the vital staining of their surroundings.	The spots are seen because of the blood in the congested skin about them.
The patches appear in skin still receiving a little blood but nearly empty of it.	The spotting is most pronounced in skin inadequately supplied with blood and nearly empty of it.
Both tend to recur in situations previously affected. Local factors which act to empty the vessels are largely responsible for this.	
The patches that have lasted long frequently persist for some time after the blood volume has been restored and the systemic blood pressure has reached the previous level or a higher one.	The spots that are most pronounced,— those usually which have lasted longest,— do not at once disappear when the circulation is restored and the surrounding skin has become actively hyperemic.

It will be seen that like causes in the two cases lead to results that are also alike in their major aspects. Whether the patches and spots are due to physiological mechanisms that are identical in detail remains uncertain. Human skin has some unique characters.

There are additional, minor points of resemblance between the two phenomena. Bier's spots are caused by a contraction of capillaries and venules, which can be followed directly under the microscope. The ischemic patching in animals cannot be so studied, but its long persistence in a skin elsewhere intensely stained after restoration of the blood bulk indicates that the small vessels must be closed down, since otherwise a staining should occur by way of the numerous vascu-

lar anastomoses. The contractile tendency is sometimes strong in Bier's spots, as shown by their development in skin engorged to the degree of ecchymosis (Tests 3 and 4). It is strong too in the ischemic patches, which frequently resist the entrance of blood at high pressure (12). Stroking will cause Bier's spots to disappear temporarily. It has the same effect on the ischemic patching, as shown by disappearance of the localized acidosis secondary to it (16).

One can exclude not a few possible causes for the contraction which gives rise to Bier's spots.

Though elicited ordinarily in an engorged skin the spots are not induced by the engorgement, but on the contrary are rendered greatly more widespread and pronounced by emptying the vessels. It follows that their cause cannot lie in changes undergone by the stagnant blood. They occur more often in "red spots," where there is still a little arterial flow, than in the violet tissue round about; and they sometimes resist the entry of arterial blood (especially when it is under very low pressure) and may rapidly appear in skin rendered pink secondarily by slowly admitting it after a period of occlusion (Test 5). From all this it would seem that neither lack of oxygen nor accumulation of carbon dioxide can be their immediate cause. Lewis' hypothesis that they are referable to highly diffusible vaso-constrictor substances developing in the tissue is, as we have shown, not tenable in its present form. The fact that the blanching is relatively infrequent in regions containing blood, and that it may be largely dissipated by a reflux of venous blood under slight pressure (Test 3) had at first seemed to bear out the idea that the fluid might have an effect to neutralize or dilute constrictor principles present in the tissues. But during the course of tests on the point it was observed, as has just been mentioned, that when blood was let slowly into tissues deprived of it for some time new spots frequently formed, and this no matter whether the fluid had come from other parts of the occluded limb or from the reopened artery. One is forced to conclude that its influence to prevent spotting, as on the under side of the arm, is merely mechanical. And this influence is not always effectual. Some definite blanchings occurred even when engorgement of the vessels was pushed beyond the tolerable limit, as shown by ecchymoses; and under such circumstances the spotting was as frequent on the upper as on the lower surface of the arm, the influence of gravity to localize it having been rendered negligible by a distension of the vessels so extreme everywhere that no shifting of their contents was possible.

Rehberg and Carrier have advanced the view that there is a greater tendency to vascular contraction at some places than at others. And certainly there are regions in which it is sufficient to overcome the maximum pressure that the vessels will stand without bursting (Tests 3 and 4). The cause is not clear for the localization of some of the resultant blanchings in the dependent skin which shows none

when the congestion is less. Perhaps the tension due to the enormous engorgement acts as a stimulus. For, as our tests have shown, the vessels liable to contract are hyperirritable, responding readily to alterations in skin tension. Whether their abnormal irritability is caused by materials retained or lacking, is uncertain, but whatever its cause filling the vessles with fresh blood does not at once do away with it. The influence of local nervous influences to condition its manifestations is far from being ruled out.

Wolf has shown that Bier's spotting occurs independently of central nervous influences (17). The fact that the situation of some of the ischemic patches in animals is frequently determined, like the spotting in man, by slight local impediments to the circulation, and the further fact that the patching thus localized may persist long after the original impediment has been rendered negligible by improvement in the general circulatory condition, both speak for the importance of local causes. Nevertheless the influence of the central nervous system must still be reckoned with. A vaso-constriction of central origin in compensation for a reduced blood bulk may cut down the peripheral circulation to such extent as to render the conditions favorable to a blanching developing secondarily. The ischemic patches on the skin of animals plainly comes about in this way. And we have shown that Bier's spots develop in man not only when stasis is complete but when there is still some little arterial flow to the part, as would be the case under the circumstances of a compensatory vaso-constriction of central origin.

One would expect Bier's spots to appear and coalesce into a widespread blanching wherever the circulation has been greatly reduced for some time. And they are actually observed. The march of events in patients with femoral embolism is as convincing on the point as any laboratory tests (18). Babies partially asphyxiated while being born are purple or mottled (*Asphyxia livida*) if the period of suffocation has not been long, but in severer instances with a feeble heart and low blood pressure the skin is waxen white (*Asphyxia pallida*) (19). These waxen babies usually cannot be revived. That adults ever live long enough in circulatory collapse for a generalized blanching to develop seems doubtful; but it appears very shortly after death, and hence the whiteness of the cadaver (20). The great pallor of the extremities in cases of severe shock or hemorrhage that have lasted for some time may well be due in part to a secondary contraction of the cutaneous vessels. Lewis (21) refers the whiteness of Raynaud's disease to this cause.

There can be no doubt that the vascular contraction which finds expression in Bier's spots and in the ischemic patching of animals is a

response highly fortunate for the organism. It acts to conserve both blood and heat, and this without expenditure on the part of the central nervous system. The gradual pace at which the flow is resumed when the ischemia has endured for a long while (22) may not be without its benefits, since thereby the accumulated metabolic products of ischemia are released only little by little into the blood stream.

In a subsequent paper the influence of the final vascular contraction upon certain forms of vaso-dilation will be discussed.

SUMMARY

A study of the blanchings (Bier's spots) which develop in human skin deprived of circulation has proved them referable to the same general causes that lead to ischemic patching of the skin of animals, and that they are conditioned by the same factors. Both are expressions of a secondary contraction of vessels that have become hyperirritable owing to an inadequate circulation. The contraction is favored by emptying the vessels, but it occurs pronouncedly even when there is a trickle through them of arterial blood. In the case of man the contractile impulse is sometimes so great as to overcome the maximum pressure that the vessels will support.

The significance of the observations is discussed.

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EXPLANATION OF PLATES

PLATE 24

FIGS. 1 and 2. To show the localization of Bier's spots to the upper side of the propped arm (see Test 1). The photographs were taken from above and in front of the arm, to include the mirror reflection of its under surface.

In Fig. 2 typical spots can be seen along the course of the radial basilic vein, which did not occur when the limb was so placed that this vessel lay on its under side (Fig. 1). (In Fig. 1 there is dimly visible on the under side of the arm a pale birthmark outlined with dots of India ink.)

PLATE 25

FIGS. 3, 4, and 5. Bier's spotting is favored by emptying the vessels and prevented by forcibly engorging them (see Test 3). The blood from the hand and lower part of the congested arm with circulation occluded had been forced into the upper part and largely retained there by means of a tourniquet.

PLATE 26

FIGS. 6, 7, and 8. Bier's spotting is favored by emptying the vessels and prevented by forcibly engorging them (see Test 4). In this subject the tendency to vascular contraction was far greater than in that of Test 3. Between the wrist and the marks of the tourniquet an immense blanched area can be seen which resisted the return of the venous blood.

PLATE 27

FIGS. 9 and 10.   The effect of local mechanical stimulation upon the vessels of the congested arm with occluded circulation (see Test 8).   The arrows point to three areas low on the side of the limb, which had been repeatedly tapped with the head of a hat-pin.   Where this struck there is a greatly increased congestion, while round about there is pallor.   Fig. 9 shows the immediate response, and Fig. 10 that it was present unchanged after 15 minutes.

PLATE 28

FIGS. 11 to 15 inclusive.   To show that Bier's spots can be made to appear by light stroking of the skin (see Test 9).   In Figs. 12 and 13 the point of the hat-pin indicates the regions previously stroked and now occupied by spots.   In Fig. 14 the blanched areas have extended and coalesced in response to strokings between them.
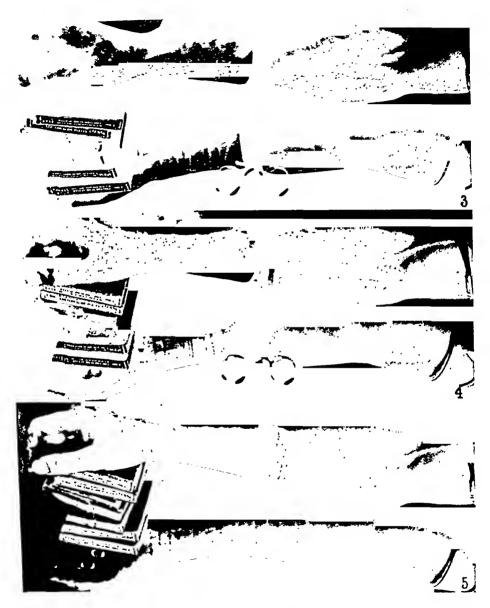
In Figs. 14 and 15 mirror reflections are included, since these demonstrate that the spotting was localized to the upper side of the arm irrespective of the position of the latter.

The photographs of Figs. 11 to 15 were taken by Cooper-Hewitt light.

(Rous and Gilding: Final response of cutaneous vessels)
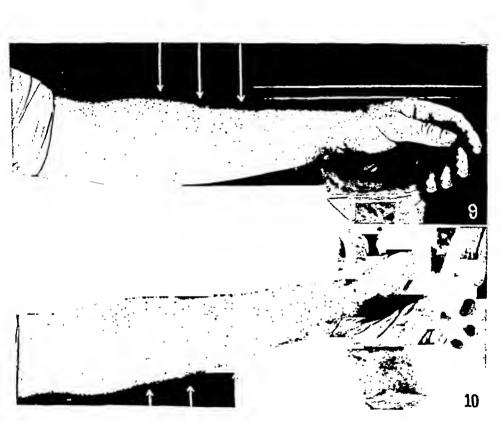
PLATE 25

(Ross and Gilling: Final response of cutaneous vessels)

6

7

8

(Ewes and Gildner: Final response of cutaneous vessels)

(Rous and Gilding: Final response of cutaneous vessels)

STUDIES ON BACILLUS TYPHOSUS TOXIC SUBSTANCES

III. The Effect of Sera upon the Injury Producing Factors of the Phenomenon of Local Skin Reactivity

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In previous communications (1–8) there was described a phenomenon of local skin reactivity to culture filtrates of bacillus typhosus and other microorganisms. The reactivity was induced by the injection of a filtrate into the skin of a rabbit. If 24 hours later an intravenous injection of the same filtrate was given to the rabbit, there appeared an extremely severe hemorrhagic necrosis at the site of the previous injection. The factors determining the local skin reactivity were termed "skin preparatory factors" and those responsible for the local injury following the intravenous injection were called "reacting factors." Features characteristic of this phenomenon as well as certain properties of the skin preparatory factors were described. One of these properties was the specific neutralizability by immune sera of the *skin preparatory* factors (2, 3). The object of the work reported in this paper was to determine the effect of sera upon the *Bacillus typhosus reacting* factors.

EXPERIMENTAL

Titration of Bacillus typhosus Reacting Factors against a Constant Amount of Skin Preparatory Factors

Filtrates of washings of 20 to 22 hour old *Bacillus typhosus* cultures on plain agar (T_L "agar washings" filtrates, Batch "A_7") were employed. The method of preparation of the filtrates has already been reported in detail (6).*

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* These filtrates were chosen in preference to those previously employed (2) because under the conditions of their preparation, there was present less extraneous material and in all probability less bacterial autolysis.

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Preliminary titrations established the fact that a dose as small as 0.25 cc. of the filtrate previously diluted to 1:100 was capable of preparing the skin of some of the rabbits to the phenomenon providing sufficiently large intravenous doses were subsequently used. Because of the high dilution, however, there occurred individual fluctuations (2, 6, 8). In order to obtain a high degree of reactivity necessary for the work to be described, rabbits were prepared with many multiples of the above indicated minimal skin preparatory dose and the reacting factors were titrated in these rabbits as follows:

Protocol I. Fifteen rabbits received each one intradermal injection of 0.25 cc. of the undiluted filtrate "A_7." Twenty-four hours later the rabbits were divided into 3 groups of five. Each group received a single intravenous injection of the same filtrate. The first group received 0.05 cc., the second group 0.01 cc. and the third group 0.005 cc., respectively, per kilo of body weight. Four and one-half hours later, there were observed severe hemorrhagic reactions in 5 rabbits of the first group, in 4 rabbits of the second group and only in 1 rabbit of the third group.

From this titration, it will be seen, that 0.01 cc. of filtrate "A_7" per kilo is the minimal dose of reacting factors capable of eliciting reactions in a large percentage of rabbits prepared with 0.25 cc. of the undiluted filtrate.

Protocol II. This experiment was identical with the experiment of Protocol I, with the exception that each of the 15 rabbits was prepared by one intradermal injection of 0.25 cc. of the filtrate "A_7" previously diluted 1:4 instead of the undiluted. In this experiment there were observed severe hemorrhagic reactions in 3 rabbits of the first group and in 1 rabbit of the second group. The third group showed no reactions.

As is seen from this experiment, 0.05 cc. per kilo is the minimal dose of reacting factors capable of eliciting reactions in a large percentage of rabbits prepared with 0.25 cc. of the filtrate "A_7" previously diluted 1:4.

For the neutralization experiments about to be described, several multiples of the above indicated minimal doses of reacting factors were employed in order to be assured of a high percentage of positive reactions in rabbits. Furthermore, inasmuch as a certain proportion of rabbits was spontaneously resistant to the phenomenon (1, 2) at least five rabbits were employed for each neutralization experiment.

One batch of the filtrate (A_7), stored in the refrigerator, was employed throughout the work. The filtrate was first used approximately two weeks after its preparation and this use was continued for two months afterwards. Since there were observed changes in the potency of different batches of filtrates from time to time, four con-

trol titrations of the reacting factors were carried out during the two months. In addition, whenever a serum neutralization experiment was carried out, a control group of 4 to 6 rabbits was injected with the filtrate alone. These experiments definitely proved that the filtrate "A₇" did not suffer any change in potency during the two months in which this work was carried out.

Neutralization of the Bacillus typhosus Reacting Factors by Specific Sera

The antityphoid sera were obtained from horses and rabbits.

The immunization of horses was carried out by simultaneous weekly subcutaneous and intravenous injections. Filtrates of 6 day old *Bacillus typhosus* cultures in tryptic digest broth, as well as toxic substances precipitated by ammonium sulphate (8) were injected subcutaneously. Unwashed *Bacillus typhosus* heat killed vaccines were injected intravenously. The length of time during which the immunization was continued before each bleeding, is indicated in following tables. The doses were increased by 25% each week. Previous to the bleeding on the eighth month, some of the horses (e.g. #2 and #4) were receiving the following rather large doses: 400 cc. of vaccine, intravenously, 1,200 cc. of filtrate, subcutaneously, a suspension of 9 grams of the precipitated toxic substances (a yield from 1,000 cc. of tryptic digest broth culture filtrate), subcutaneously, and about 400 cc. of the vaccine intravenously.

Rabbit serum R201 was a mixture of sera of 5 rabbits immunized by a method similar to that employed for horses. The injections were carried on for 4 to 6 weeks, 25 cc. of the filtrate was the largest subcutaneous dose and 20 cc. of the vaccine was the largest intravenous dose.

Normal rabbit and heterologous horse sera (Meningococcus and Erysipelas therapeutic sera) were also employed.

The experiments were conducted in a manner similar to that described in previous papers.

One area of the skin of the abdominal wall of a rabbit was injected with 0.25 cc. of the undiluted filtrate "A₇" or 0.25 cc. of the filtrate previously diluted 1:4. Twenty-two to twenty-four hours later a single intravenous injection of the filtrate or of a mixture of the filtrate with a given serum was made. The mixtures prepared on the morning of the experiments, as well as the filtrate alone were incubated in the water bath at 37°C. for one hour. The precipitate in the mixtures was broken up by shaking immediately before the injection. The readings of the reactions were made 4½ hours after the intravenous injection. A rabbit was recorded "positive" when the prepared area of the skin showed severe hemorrhagic necrosis. "Doubtful" rabbits were those showing mild punctiform hemor-

TABLE I

The Effect of Sera upon Bacillus typhosus Reacting Factors

Intradermal dose of "A₇"	Mixtures made in Vitro		Agglutination titer of sera for *B. typhosus*	Total of rabbits tested	No. of positive rabbits	No. of negative rabbits	No. of doubtful rabbits	No. of deaths
	Intravenous dose of "A₇" per kilo	Intravenous dose of serum per kilo						
0.25 cc. dil. 1:4	0.3 cc.	—	—	31	19	0	1	11
0.25 cc. undiluted	" "	—	—	57	41*	1	1	18
0.25 cc. dil. 1:4	" "	Normal rabbit ⋕282 Scrum (0.3 cc.)	1:16	19	13	0	3	3
0.25 cc. undiluted	" "	Normal rabbit ⋕169 Scrum (0.3 cc.)	Negative	5	3	0	0	2
0.25 cc. dil. 1:4	" "	Therapeutic Horse Meningococcus Scrum 148/438 (0.3 cc.) N. Y. Board of H.	1:64	9	6	2	0	1
0.25 cc. undiluted	" "	Lederle anti-Erysipelas horse serum (0.25 cc.)	Negative	15	9	0	1	5
0.25 cc. dil. 1:4	" "	**Horse anti-typhoid serum (0.3 cc.)	1:25.600	7	0	7	0	0
0.25 cc. dil. 1:4	" "	**Horse 62/4 anti-typhoid serum (0.3 cc.)	1:25.600	14	0	14	0	0
0.25 cc. undiluted	" "	**Horse 62/4 anti-typhoid serum (0.3 cc.)	1:25.600	9	0	9	0	0
0.25 cc. undiluted	" "	Horse 62/4 anti-typhoid serum diluted 1:5 (0.3 cc.)	1:25.600	7	3	4	0	0

* 4 rabbits were dead at the time of reading, but the reactions were distinct and well-defined.

** These samples of blood were taken after 7½ months of immunization.

rhagic areas of dark red appearance. In the "negative" rabbits, no reactions were seen. Some of the rabbits died in the course of the 4½ hours following the intravenous injection. Their deaths were recorded. If the dead rabbits showed

no reactions or a diffuse discoloration of the skin, no readings were recorded on the protocols. When, however, there was observed a well-defined reaction, the result was noted.

Table I represents a summary of the results obtained.

As is seen from this table, the skin and intravenous doses employed elicited severe hemorrhagic reactions in about 95% of the surviving rabbits. On the other hand, 30 rabbits which received a mixture of the same amount of the filtrate with certain immune sera (Horses 3 and 4) showed no reactions. The experiment establishes beyond question the fact that *Bacillus typhosus* reacting factors can be neutralized by immune sera.

When a mixture of the filtrate with the immune serum (Horse 4) previously diluted 1:5 was injected, there were obtained reactions in 3 rabbits out of 7. If the high percentage of positive results obtained in the control group is kept in mind, it becomes evident that the 1:5 diluted serum produced neutralization of the reacting factors but did so irregularly. The irregularity of neutralization with this dilution may be explained by assuming that the mixture contained a small amount of free reacting factors or that there was only partial neutralization of these factors so that only these rabbits possessing a high degree of reactivity were able to respond with reactions.

It will furthermore be seen that sera possessing no normal agglutinins, or agglutinins of a low titer for *Bacillus typhosus* (282, 169 and Erysipelas serum) had no neutralizing potency. With one serum (148/438 antimeningococcus horse serum) which had normal agglutinins for *Bacillus typhosus* in dilution 1:64, there was a suggestion of irregular neutralization.

The immune sera had unquestionably a remarkable protective potency against the mortality induced by the filtrate.

Titration of the Neutralizing Potency of Immune Sera

It was important further to determine whether the neutralizing potency of the sera could be quantitatively measured. In the experiments summarized in Table II, a constant amount of serum was titrated against increasing quantities of reacting factors.

As is seen from Table II, a given amount of serum was able to neutralize increasing amounts of the filtrate. The neutralization

TABLE II

Titration of the Neutralizing Potency of Anti-typhoid Sera

| Intradermal dose of "A7" | Mixtures made in Vitro | | Agglutination titer of sera for B. typhosus* | Length of antityphoid immunization | No. of rabbits tested | No. of positive rabbits | No. of negative rabbits | No. of doubtful rabbits | No. of deaths |
	Intravenous† dose of "A7" per kilo	Intravenous dose of serum per kilo							
0.25 cc. undiluted	0.5 cc.	R "201" (0.25 cc.)	1:6400	6 weeks	5	0	4	0	1
" "	0.75 cc.	" "	"	" "	5	0	2	3	0
" "	1 cc.	" "	"	" "	5	2	1	0	2
" "	0.5 cc.	Horse 2 (0.25 cc.)	1:25.600	7½ months	5	0	5	0	0
" "	1 cc.	" "	"	" "	5	0	2	2	1
" "	1.25 cc.	" "	"	" "	5	3	0	1	1
" "	0.5 cc.	Horse 5 (0.25 cc.) Bleeding 56	1:25.600	5 "	5	0	5	0	0
" "	1 cc.	" "	"	" "	5	0	5	0	0
" "	1.5 cc.	" "	"	" "	5	5	0	0	0
" "	1.5 cc.	Horse 5 (0.25 cc.) Bleeding 63	"	7½ "	5	2	3	0	0
" "	0.3 cc.	Horse 4 (0.25 cc.) Bleeding 3	1:1600**	6 weeks	5	3	2	0	0
" "	0.5 cc.	" "	"	" "	5	4	0	0	1
" "	0.75 cc.	" "	"	" "	5	2	0	1	2
" "	1 cc.	" "	"	" "	5	3	0	0	2
" "	0.5 cc.	Horse 4 (0.25 cc.) Bleeding 62	1:25.600	7½ months	15	0	15	0	0
" "	1 cc.	" "	"	" "	10	0	10	0	0
" "	1.25 cc.	" "	"	" "	5	2	3	0	0
" "	1.5 cc.	" "	"	" "	5	1	4	0	0
" "	1.75 cc.	" "	"	" "	5	2	0	1	2

* Agglutination tests were made at the time of the experiments.

** The titer was the same shortly after bleeding.

† Previous experiments have shown that these doses consistently produce reactions in animals prepared with 0.25 cc. of undiluted filtrate "A7."

was considered "consistent" if obtained in all rabbits tested (a minimum of 5 animals). As the amounts of reacting factors were increased, neutralization was obtained irregularly, i.e., in some of the

rabbits only. The maximum amount of reacting factors "consistently" neutralized by 0.25 cc. of a given serum was tentatively used to compute the neutralizing titer of a given serum. Thus, if a 0.25 cc. of a given serum is able to consistently neutralize 0.5 cc. of "A_7" (R 201, Table II) and irregularly neutralize 0.75 cc., its titer must be between these two figures. Since 0.01 cc. per kilo of reacting factors is the minimum dose (page 514), the serum has been able to neutralize between 50 and 75 minimal reacting doses, the titer, is therefore, approximately 60.

In these titrations 0.25 cc. of serum was used instead of 1.0 cc. in order to keep down the volume to be injected. The titrations in terms of 1.0 cc. must be worked out experimentally.

It will also be seen in Table II (bleedings 3 and 62, horse 4) that the titer of the neutralizing antibodies in the serum increased in the course of immunization.

As indicated in the text, the purpose of the procedure described was twofold: The first object was to establish the fact that sera can be prepared which have the power to neutralize the *Bacillus typhosus* reacting factors. The second object was to outline a method for titrating the potency of the sera. It is obvious, however, that it is necessary to develop a permanent standard for ultimate comparisons since batches of toxic substances vary in their potency. Work is under progress with this object in mind.

CONCLUSIONS AND SUMMARY

It has been demonstrated that many multiples of minimal doses of *Bacillus typhosus* reacting factors can be neutralized by specific immune sera.

The potency of a given serum can be conveniently titrated against increasing amounts of reacting factors.

If the immune serum is diluted or if the amount of the reacting factors is too large for a given amount of serum, there is obtained neutralization but only irregularly.

Normal and heterologous sera (therapeutic meningococcus and erysipelas horse sera) free of normal agglutinins or possessing normal agglutinins of a low titer (1:16) for *Bacillus typhosus* are not able to neutralize the reacting factors. There is obtained questionable

neutralization with a serum possessing normal *Bacillus typhosus* agglutinins in dilution 1:64.

The titer of the neutralizing antibodies increases in the course of immunization.

Immune sera exercise a definite protection against the mortality induced by intravenous injection of *Bacillus typhosus* culture filtrates.

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CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

I. The Synthesis of p-Aminophenol β-Glucoside, p-Aminophenol β-Galactoside, and Their Coupling with Serum Globulin

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The problems of the relationship between chemical constitution, physiological effect and biological specificity, which found their origin in the study of the active principles of certain natural drugs, have become so absorbing and so embracing that they have attracted and held the interest of the chemist, the pharmacologist, and the immunologist alike. The question of protein specificity (1), and that of whether it is possible to change specificity by altering the protein molecule through chemical means, have, in particular, engaged the minds of many investigators. On the other hand, the rôle which carbohydrates play in the phenomena of immunity has only recently been disclosed, despite the fact that the presence of these substances in bacteria and yeast has long been known.

Some years ago, Dochez and Avery (2) observed that filtrates of pneumococcus cultures contained a stable substance which reacted specifically with antipneumococcus serum of the homologous type. This observation led to the isolation and identification of these specifically reacting substances from the three specific types of Pneumococcus (3), and from the three types of Friedländer's bacillus (4). Since then, other investigators have isolated similar substances from various other species of bacteria (5).

These type specific substances fall into the class of carbohydrates. They are unusual carbohydrates in that each contains a sugar acid as an integral part of its complex molecule. Immunologically they belong to that important group of specifically reactive but non-antigenic substances which Landsteiner has termed *haptens*.

One of the striking characteristics of these bacterial carbohydrates is their failure to produce antibodies when injected into the animal organism, though in the state in which they occur in the bacterial cell they are not only type specific, but are also antigenic as well. In order that the bacterial polysaccharide may be effective as antigen it is believed, therefore, that it must be combined with another cellular constituent—possibly a protein—to form a complex and easily dissociable antigen. The nature of the substance which confers antigenicity upon the carbohydrate substance of Pneumococcus and the character of its union, are problems which as yet have not been solved.

The products of hydrolysis of these specific carbohydrates have been studied in detail (6). All except the polysaccharide of Pneumococcus Type I yield glucose on hydrolysis. The carbohydrate of Pneumococcus Type III and of Friedländer bacillus Type A yield on hydrolysis isomeric aldobionic acids (glucose-glucuronic acid) in addition to glucose. Evidence has likewise been secured which indicates that the specific carbohydrates of Type II Pneumococcus and of Type B and C Friedländer bacillus also contain other aldobionic acids.

The selective specificity of encapsulated organisms, such as Pneumococcus and Friedländer bacillus, seems to depend primarily on the hapten part of the hypothetical complex antigen. In all of the specific carbohydrates studied thus far, the invariable presence of *isomeric* aldobionic acids seems to indicate that particularly the acid group (carboxyl group) and its stereochemical relationship to other groups of the intact polysaccharide molecule, which in each instance must necessarily be different, has a profound influence in orienting specific response. This question, however, will be dealt with in greater detail in a later communication.

For the purpose of studying the rôle which simple sugars of different spatial configuration might play in altering the specificity of proteins, it was thought that it might be possible to combine these different sugars with a given protein, and to observe specific differences in antigenic properties of the substituted compounds.

The problem thus becomes two-fold, first, to find some means of combining the sugar with the protein, and second, to synthesize the appropriate derivative. Pauly's (7) fundamental method was made use of in combining glucose and galactose to serum globulin.

Some years ago Pauly (7) described a color test for the detection of tyrosine and histidine in the hydrolytic products of proteins. This test was dependent upon the formation of colored azo dyes produced by the coupling of diazobenzene sulphonic acid to the aromatic nucleus of amino acids. The coupling of diazobenzene sulphonic acid and other derivatives of phenyldiazonium chloride can be brought about not only with hydrolytic products of proteins, but with the intact protein molecule as well (8). This reaction has been used by Landsteiner (9) in preparing a number of substituted proteins,—"azo proteins,"—and in a series of experiments he studied the immunological reactions of a great variety of differently substituted proteins. By this method he was able to differentiate between the dextro and laevo forms of phenyl-amino-acetic acid. In the same communication (9f) he indicated the bearing of his observations on the serological specificity of bacterial carbohydrates.

The second problem, that of preparing a sugar derivative adaptable to this principle, was solved by the following synthesis:

$$CH_2OH—CH—(CHOH)_3—C\underset{OH}{\overset{H}{\diagup}} \quad (CH_3CO)_2O \longrightarrow$$

Glucose

$$CH_2OCOCH_3—CH—(CHOCOCH_3)_3—C\underset{OCOCH_3}{\overset{H}{\diagup}} \quad HBr \longrightarrow$$

pentacetyl
glucose

$$CH_2OCOCH_3—CH—(CHOCOCH_3)_3—C\underset{Br + Ag\cdot OC_6H_4NO_2}{\overset{H}{\diagup}} \longrightarrow$$

Tetracetylbromoglucose

Silver paranitro-
phenolate

$$CH_2OCOCH_3—CH—(CHOCOCH_3)_3—C\underset{OC_6H_4NO_2}{\overset{H}{\diagup}}$$

Tetracetyl paranitro-
phenol β-glucoside

$$\xrightarrow{\text{Ba(OH)}_2} \quad \text{CH}_2\text{OH}-\text{CH(CHOH)}_3-\overset{\displaystyle H}{\underset{\displaystyle \text{OC}_6\text{H}_4\text{NO}_2}{\text{C}}} \quad \xrightarrow{\text{H}_2}$$

paranitrophenol β-glucoside

$$\text{CH}_2\text{OH}-\text{CH}-(\text{CHOH})_3-\overset{\displaystyle H}{\underset{\displaystyle \text{OC}_6\text{H}_4\text{NH}_2}{\text{C}}}$$

paraaminophenol β-glucoside

The end product of this series of reactions may then be converted into the corresponding diazonium derivative and coupled with protein in the presence of dilute alkali:

$$\text{CH}_2\text{OH}-\text{CH}-(\text{CHOH})_3-\overset{\displaystyle H}{\underset{\displaystyle \text{O}-\text{C}_6\text{H}_4 \cdot \text{NH}_2 \cdot \text{HCl}}{\text{C}}} \quad \xrightarrow{\text{HNO}_2}$$

$$\text{CH}_2\text{OH}-\text{CH}-(\text{CHOH})_3-\overset{\displaystyle H}{\underset{\displaystyle \text{OC}_6\text{H}_4\text{N}_2\text{Cl}}{\text{C}}} \qquad + \text{ Protein}$$

$$\rightarrow \text{CH}_2\text{OH}-\text{CH}-(\text{CHOH})_3-\overset{\displaystyle H}{\underset{\displaystyle \text{OC}_6\text{H}_4\text{N}_2-\text{Protein}}{\text{C}}}$$

The most difficult step in the synthesis, the coupling of p-nitrophenol with the acetobromo-sugar, was accomplished by shaking a solution of the latter in xylene with the silver salt of p-nitrophenol. In this manner, fair yields of the glucoside

$$\text{CH}_2\text{OAc}-\text{CH}-(\text{CHOAc})_3-\overset{\displaystyle H}{\underset{\displaystyle \text{OC}_6\text{H}_4\text{NO}_2}{\text{C}}}$$

were obtained. The preparation of p-nitrophenol glucoside from this derivative, and the catalytic reduction of this compound to its amino derivative proceeded very smoothly and nearly quantitatively. The coupling of the diazonium phenol hexoside to protein takes place readily and rapidly in the presence of N/100 hydroxide.[1]

Thus with the aid of *p*-aminophenol glucoside and the corresponding galactoside two different hexosides have been attached to the same protein, yielding complexes which exhibit different optical properties and which yield reducing sugars on hydrolysis. It should be possible to attach any aldose or ketose to native protein provided the intermediary glucoside can be synthesized. Owing to the occurrence of isomeric aldobionic acids in specific bacterial polysaccharides, which are believed to play an important rôle in orienting the antigenic specificity of encapsulated bacteria, it would seem to be of even greater interest to couple sugar acids to proteins in order to study the antigenic response of such substituted proteins. We are at present attempting to synthesize such derivatives.

EXPERIMENTAL

1. *Pentacetyl glucose* and *Pentacetyl galactose*. These compounds were prepared by acetylation of the corresponding sugar with pyridine and acetic anhydride at 0°C. Both products were recrystallized from alcohol. The pentacetyl galactose melted at 146°C.

2. *Acetobromo glucose* and *Acetobromo galactose*. These derivatives were prepared by the method of Fischer (12).

3. *Silver p-nitrophenolate*. 1.2 mols of recrystallized dry p-nitrophenol were dissolved in absolute ethyl ether. 1 mol of freshly precipitated dry silver oxide was added. The mixture was shaken for 18 hours at room temperature together with a large quantity of glass heads. At the end of this time a brick red compound separated. This was filtered on a hardened paper, washed repeatedly with

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[1] There have recently appeared two papers by Klopstock and Selter (10) wherein they contend that neutral solutions of derivatives of phenyl diazonium chloride do not combine with, but are merely "adsorbed" by protein. This contention seems to have been refuted, however, by the experiments of Heidelberger and Kendall (11). It might be mentioned here again that the presence of alkali is not always necessary for the coupling of phenyl diazonium chloride derivatives to aromatic nuclei. In fact, the formation of some azo dyes is brought about by coupling diazonium chloride derivatives not only in alkaline, but in neutral, and even in acid medium as well.
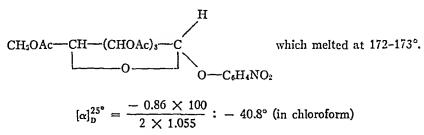
absolute ether, and dried in a vacuum desiccator over phosphorus pentoxide and paraffin for 4 weeks.   The compound is probably a mixture of the true silver salt of p-nitrophenol, $AgOC_6H_4NO_2$, and of its isomeride, $O:C_6H_4:NOOAg$.   Two distinct crystalline forms may be seen under the microscope.

Analysis: 0.2000 gms. substance gave 0.1181 gms. AgCl when analyzed by the method of Carius.

Calculated for $C_6H_4NO_3Ag$ — Ag: 43.85 per cent.   Found 44.40 per cent.

4. *Tetracetylnitrophenol β-glucoside:* 50 gms. of aceto-bromo glucose were dissolved in 500 cc. of anhydrous xylene.   To the mixture was added 10 gms. of the silver salt of p-nitrophenol.   After shaking with dry glass beads for 30 minutes a second portion of 10 gms. of the silver salt was added, and finally after another 30 minutes a third portion was added.   The mixture was removed from the shaking machine, filtered on a hardened paper, and the precipitate was washed with small portions of xylene.   The filtrate was now concentrated to 50 cc. *in vacuo.* 100 cc. of absolute ethyl alcohol were added, and then the mixture was further concentrated.   The addition of alcohol, followed by evaporation, was repeated three times.   The final concentrate was taken up in 200–300 cc. of absolute alcohol and the flask was placed in the ice box.   After 24 hours the glistening pale yellow crystals of the glucoside were filtered from the deeply colored mother liquors.   They were dissolved in about 250 cc. of hot absolute alcohol, and boiled with a little norite.   The solution was filtered through a hot water funnel.   After 24 hours in the cold the nearly snow-white crystals were filtered from the pale yellow mother liquid.   A second crystallization yielded about 9 gms. of pure glucoside,

$$CH_2OAc\text{—}CH\text{—}(CHOAc)_3\text{—}C\overset{\displaystyle H}{\underset{\displaystyle O\text{—}C_6H_4NO_2}{\diagup\diagdown}} \qquad \text{which melted at } 172\text{–}173°.$$

$$[\alpha]_D^{25°} = \frac{-0.86 \times 100}{2 \times 1.055} \; : \; -40.8° \text{ (in chloroform)}$$

Analysis: 6.595 mgs. substance: 12.370 mgs. CO_2 and 2.940 mgs. H_2O.
$C_{20}H_{23}O_{12}N$.   Calculated: C = 51.16 per cent, H = 4.94 per cent.
Found:— C = 51.15 per cent, H = 4.98 per cent.

The glucoside is readily hydrolyzed by dilute acid and yields paranitrophenol and tetracetyl glucose.   It is slowly hydrolyzed by dilute alkali in the cold.   It is insoluble in water, fairly soluble in hot alcohol.   It is readily soluble in xylene.

5. *p-Nitrophenol β-glucoside:* 20 gms. of tetracetylnitrophenol β-glucoside were shaken with 60 gms. of recrystallized barium hydroxide, dissolved in 1200 cc. of water, for 24 hours at 4°C.   At the end of this time the mixture was nearly

clear. A small amount of unchanged glucoside was filtered off. The filtrate, containing barium acetate and p-nitrophenol β-glucoside was treated with an excess of washed CO_2. The barium carbonate was centrifuged off. The supernatant liquid was concentrated to complete dryness *in vacuo*. The yellow powder was scraped from the flask and dried for two days in a high vacuum over P_2O_5. It was then extracted with 300 cc. of hot absolute alcohol and filtered from the barium acetate by using a hot water funnel. The filtrate was cooled and allowed to stand in the ice box for 2 days. The crystals formed were filtered off and were recrystallized from absolute alcohol. About 8 gms. of p-nitrophenol β-glucoside were obtained as a glistening white product, soluble in alcohol, and, to some extent, in water. This compound was readily hydrolyzed by dilute mineral acid, yielding the reducing sugar and paranitrophenol. It is slowly hydrolyzed by dilute alkali in the cold.

The substance melted at 165°C. $[\alpha]_D^{25°} = \dfrac{-1.55 \times 100}{2 \times 0.974} = -79.6°$ (in methyl alcohol).

Analysis: 4.305 mgs. substance: 7.555 mgs. CO_2 and 2.030 mgs. H_2O.

$C_{12}H_{15}O_8N$. Calculated: C 47.83 per cent   H 5.02 per cent.
Found: C 47.85 per cent   H 5.27 per cent.

6. *p-Aminophenol β-glucoside.* 5 gms. of p-nitrophenol glucoside were dissolved in 500 cc. of warm 95 per cent redistilled alcohol. To the mixture was added 0.2 gm. of platinum oxide catalyst (13). The substance was hydrogenated under atmospheric pressure in a hydrogenating apparatus. The theoretical amount of hydrogen gas, when corrected for temperature, pressure and vapor tension, was utilized in the reduction of the nitro to the amino glucoside. The reduction took place readily and quickly, and was completed within 30 minutes. The alcohol solution of p-aminophenol β-glucoside was filtered through a quantitative filter paper, and the filtrate carefully evaporated *in vacuo* to dryness. 50 cc. of absolute alcohol were added and the mixture was again evaporated to dryness. The glucoside was finally dissolved in 100 cc. of absolute alcohol and the flask was placed on ice. After 48 hours, the snow-white crystals of p-aminophenol β-glucoside were filtered from the clear mother liquors. 4.0 gms. were recovered. The compound is readily soluble in water. It is soluble in 95 per cent alcohol, though more difficultly soluble in absolute alcohol. It is readily hydrolyzed by dilute mineral acid, yielding the reducing sugar. It is also hydrolyzed by alkali in the cold. If the hydrochloride is treated with nitrous acid and the reaction mixture is poured into an alkaline solution of α-naphthol, a brilliant red dye is produced which precipitates on acidification.

The compound melts at 160–161°C. $[\alpha]_D^{26°} = \dfrac{-1.11 \times 100}{2 \times 0.867} = -64.1°$ (in methyl alcohol).

Analysis: 5.254 mgs. substance: 10.25 mgs. CO_2 and 2.925 mgs. CO_2.
$C_{12}H_{17}O_6N$.   Calculated: C 53.12 per cent, H 6.32 per cent.
                         Found: C 53.05 per cent, H 6.23 per cent.

7. *Tetracetyl p-nitrophenol β-galactoside.*   This compound was prepared exactly as was the corresponding glucoside. The yields were practically the same. It crystallizes from absolute alcohol as long glistening very pale yellow needles melting at 144–145°C.

$$[\alpha]_D^{24°} = \frac{-\ 0.17 \times 100}{2 \times 1.022} = -\ 8.3° \text{ (in chloroform)}$$

Analysis: 4.000 mgs. substance: 7.475 mgs. CO_2 and 1.685 mgs. H_2O.
$C_{20}H_{23}O_{12}N$.   Calculated: C 51.16 per cent, H 4.94 per cent
Found   C 50.96 per cent, H 4.71 per cent

8. *p-Nitrophenol β-galactoside.*   This derivative was prepared exactly as was the glucoside.   25.4 gms. of p-nitrophenol tetracetyl β-galactoside yielded 11.9 gms. of p-nitrophenol β-galactoside. The compound crystallizes from absolute alcohol as colorless needles melting at 181–182°C.

Analysis: 4.100 mgs. substance: 7.175 mgs. CO_2 and 1.825 mgs. H_2O
$C_{12}H_{15}O_8N$.   Calculated: C 47.83 per cent, H 5.02 per cent.
                         Found   C 47.72 per cent, H 4.99 per cent.

9. *p-Aminophenol β-galactoside.*   The reduction of p-nitrophenol β-galactoside was carried out in the same way as was that of the corresponding glucoside.   The reduction does not seem to take place quite as rapidly as does that of the glucoside. The yields are practically quantitative.   The compound was isolated from absolute alcohol as a white crystalline product soluble in water.   It suffers hydrolysis when boiled with dilute mineral acid.   When hydrolyzed solutions of the amino gluco-side are boiled with Fehling's solution, a powerful reduction takes place.

The substance melts at 158–159°C. $[\alpha]_D^{25°} = \dfrac{-\ 74 \times 100}{2 \times 0.914} = -\ 40.5°$ (in methyl alcohol).

Analysis: 5.350 mgs. substance: 10.370 mgs. CO_2 and 3.160 mgs. H_2O.
$C_{12}H_{17}O_6N$.   Calculated: C 53.12 per cent, H 6.32 per cent.
                         Found: C 52.85 per cent, H 6.61 per cent.

10. *Preparation of Serum Globulin.*   1 liter of normal horse serum was poured into 15 liters of distilled water.   Dilute acetic acid was added until the maximum turbidity occurred.   The mixture was placed in the ice chest for 48 hours.   The supernatant liquid was then syphoned off and discarded.   The precipitate was centrifuged in the cold.   The globulin thus obtained was dissolved in physiological

salt solution and reprecipitated. A final globulin solution of 2 per cent concentration was employed for coupling with the glucosides.

11. *Preparation of Protein-diazophenol glucoside and galactoside.* 100 cc. of 2 per cent globulin solution were placed in a flask and cooled to 0°C. 100 cc. of N/50 NaOH, in physiological salt solution, were also cooled to 0°C. Then 1.00 gm. of p-aminophenol β-glucoside was dissolved in 25 cc. of water. 2 mols of N/1 hydrochloric acid were added. The mixture was cooled to 0°C. The theoretical quantity of sodium nitrite, dissolved in 10 cc. of water, was slowly added. After diazotization was complete and the solution showed no excess of nitrite, it was slowly added to the globulin, which had previously been mixed with the chilled alkali. A yellow color immediately developed which soon deepened to a dark wine-red. After standing for 30 minutes at 0° the mixture was treated with 50 cc. of chilled N/50 HCl in physiological salt solution. A precipitate of highly colored insoluble protein flocculated from the mixture. This protein was centrifuged from the colored supernatant liquid. The precipitate was then thoroughly extracted five times with a total of one liter of 3 per cent salt solution. The final washing was nearly colorless and protein free. The precipitate of protein diazophenol glucoside was suspended in 100 cc. of physiological salt solution and N/20 sodium hydroxide was cautiously added with stirring, until the suspension was completely in solution. The total dry weight of material was determined by evaporation, after dialysis, *in vacuo* to constant weight. The optical rotation was determined in a one-half decimeter tube using the wave length 760.8 mm. which has the same color as the azo-protein. The galactose diazophenol protein was prepared in exactly the same way as was the glucose derivative. It was finally taken up in 200 cc. of physiological salt solution.

Properties of the synthetic sugar proteins

Optical Rotation: A solution of the diazophenol glucoside protein, containing 9.725 mgs. of ash-free solid per cc., rotated the plane of polarized light −0.21° in a 0.5 decimeter tube.

$$[\alpha]^{25°}_{760.8\,\mu\mu} = \frac{-0.21 \times 100}{0.5 \times 0.9725} = -43.2°$$

A solution of the diazophenol galactoside protein, containing 5.200 mgs. of ash-free substance per cc., gave an observed rotation of −0.08° in a 0.5 decimeter tube.

$$[\alpha]^{25°}_{760.8\,\mu\mu} = \frac{-.08 \times 100}{0.5 \times 0.5200} = -30.7°$$

Owing to the difficulty of securing a bright field of vision through these deeply colored solutions, the observed rotations probably suffered from an appreciable error. Six successive readings checked within 0.03°.

Reducing Sugars on Hydrolysis:—2 cc. of the protein solutions of the above concentration were placed in glass tubes together with 2 cc. of N/1 HCl. The tubes were sealed and placed in a boiling water bath. At the end of 3 hours and 5 hours respectively the tubes were removed, cooled, and centrifuged. A sugar analysis was made on 2 cc. of the hydrolysate by the method of Van Slyke and Hawkins (14). The galactose protein yielded approximately 10 per cent of reducing sugars, calculated as glucose. The glucose-protein yielded approximately 17 per cent of reducing sugars calculated as glucose.[2] There was no difference in the reducing value of the samples hydrolyzed for 3 hours when compared with those hydrolyzed for 5 hours.

The two synthetic sugar proteins appear to be different compounds. Animals have been immunized with these synthetic sugar proteins. The immunological results are reported in the following communication.

SUMMARY

1. The synthesis of p-aminophenol β-glucoside and p-aminophenol β-galactoside has been described.

2. These hexosides have been coupled to serum globulin. Two protein sugar complexes with different optical properties have been obtained.

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I

CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

II. Immunological Specificity of Synthetic Sugar-Protein Antigens

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The function of carbohydrates as determinative substances in bacterial specificity is well illustrated in the immunological differentiation of the specific types of pneumococci and of Friedländer bacilli. Previous studies have demonstrated that the presence of type-specific polysaccharides in these encapsulated organisms determines the antigenic properties of the cell and the serological relationships of different strains.

In the course of investigations on the chemo-immunological nature of these complex bacterial antigens, the facts thus far ascertained lend support to the view that the specific polysaccharides function as true antigens only when combined with some other constituent of the cell. While the character of the substance which enters into combination with these complex sugars, and the nature of the linkage between them are still undetermined, it seems probable that the type-specific antigen consists of a protein or protein derivative conjugated with the specific carbohydrate; and that it is the latter component which orientates the specificity of the complex antigen thus formed. The possibility also exists that the polysaccharides which by themselves are non-antigenic, may, by reason of their acidic properties acquire antigenicity by combining to form salts with some basic constituent of the bacterial cell. This latter possibility, as well as the supposition that a mere change in the physical state of the polysaccharide as it exists in the intact cell may account for its antigenic behavior, seem in the light of present knowledge, to be

less likely than does the former view that the type-specific antigen consists of a conjugated carbohydrate-protein complex in which the carbohydrate radical determines the specificity of the whole.

With a view to the possible elucidation of the problems just stated, it appeared of considerable interest to determine the effect produced on the antigenic specificity of a protein by combining it with a relatively simple carbohydrate. This combination was effected, as described in the preceding paper (1), by synthesis of the p-aminophenol glucosides of glucose and galactose, and by coupling these diazotized glucosides with different proteins.

The experiment was made all the more exacting by the purposeful choice of two monosaccharides which have the same chemical formula and which differ from each other only in specific rotation and molecular configuration:—the groups on the fourth carbon atom in galactose forming the mirror image of the fourth carbon groups in glucose. The remainder of the molecule is the same in both sugars. The relationship of the p-aminophenol β-glucoside to p-aminophenol β-galactoside may be seen from the two structural formulae—

p-aminophenol β-glucoside . p-aminophenol β-galactoside

The diazonium derivatives of these two glucosides were attached to proteins by means of the linkage $-N = N - \langle \rangle -$ and the two synthetic sugar proteins thus derived were used as antigens.

The specificity of these conjugated carbohydrate-proteins was further tested by linking the same diazotized glucoside to two chemically distinct proteins derived from widely remote biological species.

It will be shown that immune sera prepared with these complex

antigens contain two separate kinds of antibodies; one variety stimulated by the conjugated sugar-protein, and the other evoked by the protein itself, varying amounts of which coexist unbound in the same solution. The immunological specificity of the synthetic sugar-proteins described in the present paper will be discussed, therefore, with reference to these two kinds of antibodies; 1. the anticarbohydrate antibodies (anti-S) and 2. the antiprotein antibodies (anti-P.). It will be shown further that each variety of antibody is specifically related to the corresponding component of the antigen; that the antiprotein antibodies exhibit the species specificity of the original protein, and that the antibodies reactive with the conjugated sugar-proteins are specific for unrelated proteins containing the same diazotized carbohydrate group.

The results of cross precipitin reactions, precipitin absorption and inhibition tests with immune sera prepared with the synthetic sugar-proteins are presented in the following protocols.

<center>EXPERIMENTAL</center>

Methods

Rabbits were immunized by the intravenous injection of solutions of the conjugated carbohydrate-protein antigens. The animals of one series received the antigen composed of purified globulin from horse serum coupled to diazo phenol glucoside, and those of another series were treated with the same protein combined with the diazo phenol galactoside. The rabbits of both series were injected with 2 cc. of the respective antigen daily for six doses and the course of injections was repeated at weekly intervals until a total of 36 cc. of antigen was given. Eight days after the last injection the rabbits were bled and the serum tested for precipitins against the homologous and heterologous antigens.

The antigenic material was prepared by the method described in the preceding paper, and was sterilized by passage through a Berkefeld filter. The same stock solution was used throughout the course of immunization. None of the rabbits showed any evidence of toxic symptoms following repeated injections.

Preparation of proteins:—The two proteins to which the diazo phenol glucosides were linked were serum globulin (horse) and crystalline egg albumin. The globulin was prepared from horse serum by precipitation with dilute acetic acid as described in the preceding paper. The crystalline egg albumin was made from native egg white by the method of Soerensen (2).

Preparation of carbohydrate-proteins:—By the methods described in the preceding paper (1) the following synthetic sugar-proteins were prepared:

1. phenol β glucoside-azo-globulin.
2. phenol β galactoside-azo-globulin.
3. phenol β glucoside-azo-albumin.
4. phenol β galactoside-azo-albumin.

For the sake of convenience the above preparations will be referred to respectively as gluco-globulin, galacto-globulin, gluco-albumin, and galacto-albumin.

Precipitin reactions:—The immune sera were in all instances used in constant amounts of 0.2 cc. A dilution of serum in the proportion of 2 parts of serum to 3 parts of salt solution was prepared and 0.5 cc. of this dilution, containing 0.2 cc. of the original serum, was added to 0.5 cc. amounts of the varying dilutions of the antigens as shown in the protocols.

Standardization of antigens:—For purposes of comparison, the sugar protein antigens were standardized on the basis of nitrogen-content; this method, however, does not indicate the amount of bound carbohydrate and hence is not a measure of the effective antigen complex.

I. ANTICARBOHYDRATE ANTIBODIES: (ANTI-S)

1. Precipitin reactions with Gluco-globulin Anti-Serum

Serum prepared by immunization with gluco-globulin was tested for the presence of precipitins against the homologous antigen and two other sugar-proteins, gluco-albumin and galacto-albumin. Both of the latter test substances contain egg albumin, a protein foreign to the immunizing antigen. In the case of gluco-albumin, however, the carbohydrate radical is the same as that present in the sugar-protein used for immunization, while in the case of galacto-albumin both the protein fraction and the sugar derivative are heterologous with respect to the original antigen. The results of cross precipitin tests are given in Table I.

The data presented in Table I show that gluco-globulin anti-serum reacts not only with gluco-globulin but also with gluco-albumin. The fact that antibodies stimulated by gluco-globulin are specifically reactive with gluco-albumin, which contains the homologous glucose derivative bound to a protein unrelated to that present in the immunizing antigen, demonstrates that the carbohydrate radical and not the protein molecule orientates the specificity of these conjugated sugar-proteins. The specificity of the orienting carbohydrate is further emphasized by the fact that antibodies reactive with gluco-albumin show no reaction with galacto-albumin in which an isomeric carbo-

hydrate is conjugated with the same protein. This is further proof that the chemical constitution of the sugar radical, regardless of the nature of the protein to which it is attached, determines the serological specificity of the conjugated antigen.

TABLE I

Precipitin Reactions of Gluco-Globulin Antiserum

Showing the specificity of sugar-proteins when the same carbohydrate derivative is combined with two serologically distinct proteins.

Anti-Gluco-Globulin Serum

Dilutions of antigens†	Carbohydrate-Protein Antigens		
	Gluco-globulin (horse)	Gluco-Albumin (egg)	Galacto-Albumin (egg)
1:1000	++±	++	~
1:5000	++++	++++	~
1:10,000	++++	++++	~
1:20,000	+++±	+++	~
1:40,000	+++	+	~
1:80,000	+	±	~
1:100,000	±	—	~

++++ = Complete precipitation with clear supernatant and formation of a compact, disc-like precipitate not easily disrupted by shaking— characteristic of type specific polysaccharide reactions with anti-pneumonococcus serum.

± = Faint turbidity.

~ = No reaction.

† The antigens were standardized on the basis of nitrogen content. This method does not measure the amount of glucoside bound to protein.

2. *Precipitin reactions with Galacto-Globulin Anti-serum*

The cross precipitin reactions with galacto-globulin antiserum and three synthetic sugar-proteins, galacto-globulin, galacto-albumin and ʃgluco-albumin, are given in Table II.

Specific precipitins in the serum of rabbits immunized with galacto-globulin react with solutions of galacto-globulin and galacto-albumin in approximately equal titre. These antibodies, however, fail to precipitate gluco-albumin. (Table II.) The specificity of the orienting sugar radical is again revealed in the cross precipitin reac-

tions between galacto-globulin and galacto-albumin. The carbo-
hydrate derivative alone is common to both of these compounds, while
the protein fraction of each is wholly dissimilar. The specific relation-
ships between these sugar-proteins, therefore, appear to depend upon
the nature of the particular carbohydrate component rather than upon
the kind of protein to which it is linked.

TABLE II

Precipitin Reactions of Galacto-Globulin Antiserum

Showing the specificity of anti-S antibodies by cross-reactions with two sero-
logically distinct proteins containing the same sugar-radical.

Anti-*Galacto*-Globulin Serum

Dilution of Antigen	Antigens		
	Galacto-Globulin	Galacto-Egg-Albumin	Gluco-Egg-Albumin
1:5000	++++	+++±	—
1:10,000	++++	+++±	—
1:20,000	+++	+++	—
1:40,000	++±	++	—
1:80,000	++	+	—
1:100,000	+	±	—

++++ = Complete precipitation with compact, disc-like precipitate.
— = No reaction.

The data presented in Table I and II may be summarized briefly as
follows:—

1. When two chemically different carbohydrate derivatives are
bound to the same protein, the newly formed compounds are sero-
logically distinct from one another. Simple differences in the molec-
ular configuration of the two isomers, glucose and galactose, although
confined to one single carbon atom in the molecule, suffice to orien-
tate antigenic specificity when corresponding glucosides of these
sugars are coupled to the same protein.

2. When the same carbohydrate radical is conjugated with two
chemically and serologically distinct proteins, both of the sugar-
proteins thus formed acquire a common serological specificity.

3. The newly acquired specificity of these sugar-proteins is deter-
mined by the chemical constitution of the carbohydrate attached to
the protein molecule.

3. Specific Inhibition of Precipitin Reactions by Homologous Glucosides

In his studies on complex antigens Landsteiner (3) has shown that immune sera prepared with azo-proteins are markedly specific, precipitating unrelated proteins which contain the same azo groups. However, the simple azo compounds by themselves are not antigenic and are not precipitable in azo-protein antiserum. Nevertheless, when they are added to the immune serum prepared with protein coupled to the same diazotized compounds they specifically inhibit the antibodies from reacting subsequently with the homologous antigen.

Since in the present study the diazotized phenol glucosides of glucose and galactose exhibit immunological properties analogous to those of the diazotized amino compounds described by Landsteiner, the inhibiting action of these carbohydrate substances on the precipitins of homologous immune sera was studied.

Inhibition Test: Solutions of p-aminophenol β-glucoside and p-aminophenol β-galactoside in concentrations of 0.1 M. and 0.01 M. were added in amounts of 0.2 and 0.1 cc. to a constant unit of immune serum (0.2 cc.) and made up to volume by addition of salt solution. The mixtures were incubated at 37°C. for 2 hours, and the tubes examined for the presence of precipitate. To the test mixtures, 0.5 cc. of sugar protein antigen containing the homologous carbohydrate derivative was then added in optimal dilution, and the tubes again incubated for 2 hours. Readings were made at the end of the period of incubation and after 24 hours in the ice-box.

The results of the inhibition tests with homologous glucosides are given in Tables III and IV.

Since the inhibiting action of the homologous glucosides on specific precipitins is similar in each instance, the results will be discussed together. (Tables III and IV.) It is apparent that the glucosides by themselves are not precipitated in the presence of immune sera prepared with protein containing the homologous diazotized compounds.

It is also evident that although the carbohydrate substances alone fail to cause precipitation in immune serum, they, nevertheless, specifically inhibit the precipitating antibodies from reacting with the homologous sugar-protein when the latter is subsequently added to the serum mixture. The specificity of the reaction is shown by the fact that while the addition to homologous serum of 0.1 cc. of 0.01 M solution of the corresponding glucoside completely inhibits precipitation

when the specific carbohydrate-protein is added later, the addition of a ten to twenty-fold concentration of a heterologous glucoside, under the same conditions, exerts no inhibition on the precipitating antibodies.

TABLE III

Specific Inhibition by Homologous Glucoside of Precipitin Reactions with Gluco-Globulin Serum

	Gluco-Globulin Serum	Glucoside		Galactoside		Salt solution to volume	Incubation	Antigen Gluco-Egg Albumin 1:10,000	Result	
		0.1 M.	0.01 M.	0.1 M.	0.01 M.				2 hrs. 37°C.	24 hrs. ice-box
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*		*cc.*		
1	0.2	0.2	—	—	—	0.3		0.5	—	—
2	0.2	0.1	—	—	—	0.4		0.5	—	—
3	0.2	—	0.2	—	—	0.3	Incubation—2 hrs. at 37°C. No precipitate formed.	0.5	—	±
4	0.2	—	0.1	—	—	0.4		0.5	±	+
5	0.2	0.2	—	—	—	0.8		—	—	—
6	0.2	—	—	—	—	0.5		0.5	++	++++
7	0.2	—	—	0.2	—	0.3		0.5	++	++++
8	0.2	—	—	0.1	—	0.4		0.5	++	++++
9	0.2	—	—	—	0.2	0.3		0.5	++	++++
10	0.2	—	—	—	0.1	0.4		0.5	++	++++
11	0.2	—	—	0.2	—	0.8		—	—	—
12	0.2	—	—	—	—	0.7		—	—	—
13	—	—	—	—	—	0.7		0.5	—	—
14	—	0.2	—	—	—	1.0		—	—	—
15	—	—	—	0.2	—	1.0		—	—	—

+++ = Complete precipitation.
— = No reaction.

The simple monosaccharides,—glucose and galactose—from which the respective glucosides were synthesized, have been found to be inert in specific precipitin and inhibition reactions with immune sera prepared with protein united to the corresponding diazotized glucosides.

The data presented in Tables III and IV, may be summarized as follows:—

1. The unconjugated glucosides, although themselves not precipitable in immune serum, inhibit the reaction between the homologous sugar-protein and specific antibody.

2. This inhibition is specific, since heterologous glucosides exert no inhibiting action.

TABLE IV

Specific Inhibition by Homologous Galactoside of Precipitin Reactions with Galacto-Globulin Serum

	Galacto-Globulin Serum	Galactoside		Glucoside		Salt solution to volume		Antigen Galacto-Egg Albumin 1:10,000	Result	
		0.1 M.	0.01 M.	0.1 M.	0.01 M.				2 hrs. 37°C.	24 hrs. ice-box
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*		*cc.*		
1	0.2	0.2	—	—	—	0.3		0.5	—	—
2	0.2	0.1	—	—	—	0.4		0.5	—	—
3	0.2	—	0.2	—	—	0.3		0.5	—	±
4	0.2	—	0.1	—	—	0.4		0.5	±	±
5	0.2	0.2	—	—	—	0.8		—	—	—
6	0.2	—	—	—	—	0.5		0.5	++	+++
7	0.2	—	—	0.2	—	0.3		0.5	++	+++
8	0.2	—	—	0.1	—	0.4		0.5	++	+++
9	0.2	—	—	—	0.2	0.3		0.5	++	+++
10	0.2	—	—	—	0.1	0.4		0.5	++	+++
11	0.2	—	—	0.2	—	0.8		—	—	—
12	0.2	—	—	—	—	0.7		—	—	—
13	—	—	—	—	—	0.7		0.5	—	—
14	—	0.2	—	—	—	1.0		—	—	—
15	—	—	0.2	—	—	1.0		—	—	—

Incubation—2 hrs. 37°C. No precipitate formed.

3. These glucosides exhibit the properties of haptens; they are non-antigenic substances which are specifically reactive, as shown by inhibition tests, with antibodies induced by proteins containing the homologous diazotized compounds.

II. ANTIPROTEIN ANTIBODIES (ANTI-P)

In the preceding experiments the immune sera prepared with conjugated sugar-proteins have been analyzed only with reference to

the presence of antibodies specifically related to the particular carbo-
hydrate radical introduced into the protein molecule.   These so-called
anti-carbohydrate antibodies (Anti-S) are specifically reactive with
unrelated proteins containing the same diazotized glucosides.   How-
ever, in addition to the antibodies just described, the sera also contain

TABLE V

Precipitin Reactions of Gluco-Globulin Antiserum

Showing the species specificity of the antiprotein antibodies in cross reactions
with antigens containing the same protein molecule.

Gluco-Globulin Serum

Dilution of Antigens	Carbohydrate-protein Antigens					
	Gluco-Globulin (horse)	Galacto-Globulin (horse)	Globulin (horse)	Gluco-Albumin (egg)	Galacto-Albumin (egg)	Albumin (egg)
1:5000	$\left(\frac{++++}{xxxx}\right)$	xxxx	xxxx	$+++\pm$	—	—
1:10,000	$\left(\frac{++++}{xxxx}\right)$	xxx	xxxx	$+++$	—	—
1:20,000	$\left(\frac{+++}{xxx}\right)$	xx	xx	$++\pm$	—	—
1:40,000	$\left(\frac{+++}{xxx}\right)$	x	$\underline{xx}$	$+\pm$	—	—
1:80,000	$\left(\frac{+}{x}\right)$	$\underline{x}$	x	$\pm$	—	—
1:100,000	$\left(\frac{\pm}{x}\right)$	—	$\underline{x}$	—	—	—

x = Reactions with common species protein by Anti-P antibodies.
+ = Reactions with specific carbohydrate group by Anti-S antibodies.

anti-protein antibodies which are reactive only with the particular
kind of protein present in the immunizing antigen.   The following
experiments deal with the occurrence, the species specificity and
specific absorption of the anti-protein (Anti-P) antibodies.

1. Occurrence: In the conjugated antigens used for immunization

the total amount of protein present was estimated by determining the nitrogen content of the solution. However, this method of standardization is obviously inadequate, since it provides no measure of the relative amount of protein bound by the diazotized glucoside. As an excess of protein was always used for coupling with the carbohydrate, a greater or less amount of unbound protein remained free in solution depending upon the variable factors involved in the chemical reactions

TABLE VI

Precipitin Reactions of Galacto-Globulin Antiserum

Dilution of Antigen	Galacto-Globulin	Gluco-Globulin	Globulin	Gluco-Albumin	Galacto-Albumin	Albumin
1:5000	$\left(\begin{array}{c}++++\\x\end{array}\right)$†	$\underline{x}$	$\underline{x}$	—	++++‡	—
1:10,000	$\left(\begin{array}{c}++++\\x\end{array}\right)$	$\underline{x}$	$\underline{x}$	—	++++	—
1:20,000	$\left(\begin{array}{c}+++\\x\end{array}\right)$	x	—	—	++±	—
1:40,000	$\left(\begin{array}{c}++\\x\end{array}\right)$	$\underline{x}$	—	—	++	—
1:80,000	$\left(\begin{array}{c}+\\x\end{array}\right)$	—	—	—	+	—
1:100,000	$\left(\begin{array}{c}x\end{array}\right)$	—	—	—	—	

† = Bulky precipitate representing combined action of Anti-S and Anti-P.
‡ = Compact disk-like precipitate characteristic of Anti-S reactions.

associated with the process of diazotization. The occurrence of antiprotein antibodies in the serum of rabbits immunized with glucoglobulin and galacto-globulin is, therefore, presumably due to the presence in both antigens of an excess of common protein free in solution.

The differences observed in the titre of antiprotein precipitins in the two sera, (Tables V and VI) appear to be attributable to corresponding differences in the relative amount of free protein in each

antigen. This opinion is strengthened by the fact that the gluco-globulin antigen which yielded a high concentration of Anti-P precipitins was found on analysis to contain approximately 63 per cent more protein by weight than was present in the solution of galacto-globulin. This difference is reflected in the relative concentration of precipitins, since the serum prepared with the antigen having the greater amount of protein contains the higher concentration of anti-protein antibodies.

In this connection it is interesting to observe that, despite the differences in anti-protein response, both antigens gave rise to specific anticarbohydrate antibodies in approximately equal titre.

TABLE VII

Precipitin Reactions of Anti-horse Serum with Sugar-proteins containing horse serum Globulin

Anti-horse Serum

Dilution of Antigen	Gluco-Globulin	Galacto-Globulin	Original Globulin
1:1000	xx	xx	xxx
1:5000	xxxx	xxxx	xxxx
1:10,000	xxx	xxx	xxxx
1:20,000	xx	xx	xxx
1:40,000	x	x	xx
1:80,000	x	x	x

The antiprotein antibodies evoked by immunization with gluco-globulin and galacto-globulin, are presumably due to the presence in the antigens of free protein unbound by the diazotized glucosides. The supposition that, even in the absence of free protein, the sugar-protein compex alone may give rise to two qualitatively different antibodies, each specifically related to the corresponding constituent of the antigenic complex, affords an interesting alternative explanation of the occurrence of the two antibodies in the same immune serum. This concept involves the assumption that the binding of the diazo-glucoside to the protein does not entirely mask the groups essential to the specificity of the protein molecule, and that the carbohydrate radical through conjugation acquires specific antigenicity while the protein molecule retains in part its original antigenic properties.

2. *Specificity:* The antiprotein antibodies commonly found in these

immune sera exhibit only the species specificity of the particular kind of protein present. Since the same protein was used in preparing both test antigens, it is not surprising that a serum obtained with one shows cross precipitation with solutions of the other. The antiprotein precipitins in both sera react only in the presence of the homologous protein of the same species. They do not precipitate proteins unrelated to that present in the immunizing antigen. In this respect they are easily differentiated from the anticarbohydrate antibodies which, as shown in the present study, react with heterologous protein containing the same sugar radical.

TABLE VIII

Differentiation of Anti-S and Anti-P Antibodies by Specific Inhibition with Homologous Glucoside

Gluco-horse-Globulin Serum from rabbit*	Antigens							
	Gluco-Albumin (Egg)†				Globulin (Horse)‡			
	1:5000	1:10,000	1:20,000	1:40,000	1:5000	1:10,000	1:20,000	1:40,000
With the addition of Glucoside..........	—	—	—	—	xx	xx	xx	x
Without the addition of Glucoside..........	+++±	++++	+++	+±	xxx	xx	xx	x

* Precipitating serum prepared by immunization of rabbit with solution of glucoside coupled to globulin obtained from horse serum.

† Demonstrating anti-carbohydrate (anti-S) reactions.

‡ Demonstrating anti-protein (anti-P) reactions.

The species specificity of the antiprotein precipitins is demonstrated in Tables V and VI. The species relationship between homologous protein and its antibody is again illustrated in Table VII, in which it is evident that a precipitating serum prepared by immunization of rabbits with plain horse serum reacts with solutions of gluco- and galacto-globulin containing in common a native protein of the same animal origin. These protein-antiprotein reactions conform in all respects to the well known principles of species specificity. They are included here only to emphasize by contrast the preceding observations on the new specificity acquired by proteins of unrelated species when a simple sugar radical is attached to the protein molecule.

derivatives of two different sugars the resulting compounds are, in each instance, serologically distinct and specific.

In addition, the present work reveals the important fact that derivations of the two simple monosaccharides—glucose and galactose—which differ one from the other only in the spatial configuration of a single carbon atom, exhibit distinct immunological specificity when combined with the same or different proteins. Thus, for the first time, it has been shown by direct experimental evidence that asymmetry of the carbon atoms in the sugar radical alone suffices to determine differences in the specificity of sugar-protein antigens. Furthermore, these simple carbohydrate substances mask the species specificity of the protein molecule to which they are attached, and confer upon the combined antigen a new specificity which is dependent solely upon the chemical structure of the simple sugar radical.

The particular hexosides used in the present study were synthesized from glucose and galactose in form of the corresponding p-aminophenol β-glucoside and galactoside. These substances, when injected into the animal body, apparently do not stimulate the formation of demonstrable antibodies in the blood serum of the treated rabbits. At least it may be stated that attempts to induce antibody formation have failed despite the fact that both glucosides contain approximately five per cent of nitrogen. This lack of ability to stimulate the formation of antibodies is interesting in the light of Ford's original observation (5) that a nitrogenous glucoside isolated from a variety of poisonous mushroom produced antibodies which neutralized the hemolytic action of the glucoside. It may be mentioned in passing that the glucosides used in the present study are not hemolytic for rabbit blood cells. Moreover, both glucosides fail to cause the formation of a precipitate when added to an immune serum prepared with the homologous sugar-protein. The lack of specific precipitation in immune serum may be referable to the fact that the simple sugar derivatives are crystalloids and of relatively small molecular size when compared with the colloidal and highly complex sugars of Pneumococcus which react so readily in precipitin tests with specific antibacterial sera. However, despite the lack of specific precipitability, the homologous glucoside when added to the test mixture inhibits the precipitin reaction between the corresponding sugar-protein and its specific antibody.

The mechanism of the inhibition is not as yet clear. It is definite, however, that the inhibitory action of the sugar radical is specific. The precipitins for galacto-protein compounds, for example, are inhibited only in the presence of the homologous galactoside and are not affected by the addition of the heterologous glucoside (Tables III and IV). Moreover, the inhibition reaction, as Landsteiner has pointed out in the case of the azo-proteins, affords a specific method for the serological differentiation of simple chemical substances,—in this instance isomeric sugar derivatives—which by themselves are not antigenic and non-precipitable in immune sera. By reason of their serological specificity it appears justifiable to place these artificially prepared glucosides in the class of carbohydrate haptens, the most conspicuous examples of which are the specific polysaccharides naturally found in certain micro-organisms.

The results obtained with these synthetic sugar-proteins offer suggestive lines of approach to a further study of the chemo-immunological nature of those complex bacterial antigens in which carbohydrates are known to be the specific substances.

SUMMARY

1. When two chemically different carbohydrate derivatives are bound to the same protein, the newly formed antigens exhibit distinct immunological specificity.

2. When the same carbohydrate radical is conjugated with two chemically different and serologically distinct proteins both of the sugar-proteins thus formed acquire a common serological specificity.

3. The newly acquired specificity of the artificially prepared sugar-proteins is determined by the chemical constitution of the carbohydrate radical attached to the protein molecules. Simple differences in the molecular configuration of the two isomers,—glucose and galactose—suffice to orientate protein specificity when the corresponding glucosides of the two sugars are coupled to the same protein.

4. The unconjugated glucosides, although themselves not precipitable in immune serum, inhibit the reaction between the homologous sugar-protein and its specific antibody. The inhibition test is specific.

5. The sugar derivatives unattached to protein exhibit the properties of carbohydrate haptens; they are non-antigenic but specifically

reactive, as shown by inhibition tests, with antibodies induced by proteins containing the homologous diazotized glucoside.

6. The specificity of artificially prepared sugar-proteins is discussed with reference to the chemo-immunological nature of the bacterial antigens containing complex sugars.

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CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

III. ACTIVE AND PASSIVE ANAPHYLAXIS WITH SYNTHETIC SUGAR-PROTEINS

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In the two preceding papers of this series Avery and Goebel (1, 2) have reported the results of chemo-immunological studies on conjugated carbohydrate-proteins.

In the first of these communications Goebel and Avery (1) described the chemical methods by means of which the p-aminophenol glucosides were synthesized from glucose and galactose and the diazotized substances bound to protein. The two newly synthesized sugar-proteins differ from one another chemically only in specific rotation and molecular configuration. In the second paper, Avery and Goebel (2) reported the antigenic properties and serological specificity of the conjugated carbohydrates. They found that the glucosidic radical of the compound antigen endowed the new complex with specific reactivity. This was demonstrated in two ways. First, when the *same* glucoside is attached to two *different* proteins, as serum globulin or egg albumin, the serum prepared by immunization with either one of the antigens is specifically reactive with the other. Second, when the two *different* glucosides,—glucoside and galactoside—are each combined with the *same* protein, the newly formed compounds are serologically distinct. These facts were found to be true even though the individual glucosides are isomers differing only in the spatial configuration of a single carbon atom.

On the other hand the uncombined glucosides alone were found to be non-antigenic. They failed to induce antibody formation in the animal body and caused no visible precipitation when added to immune sera *in vitro*. However, both glucosides possess the capacity of specifically inhibiting the precipitating action of homologous antisugar-protein serum. When galactoside was mixed with serum prepared by immunization with galacto-globulin,[1] the subsequent addition

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[1] The terms employed in this paper to represent the sugar-protein compounds are the same as those used by Avery and Goebel (2).

of galacto-albumin to the mixture did not cause precipitation.   Furthermore, when the heterologous glucoside was substituted in the same system, no inhibition of the precipitin reaction occurred.   These results demonstrate the specificity of the inhibition phenomenon.   Avery and Goebel (2) considered that the uncombined glucosides possess the immunological properties of haptens.

The rôle of carbohydrate in anaphylaxis has been a subject of recent experimental investigation.

Tomcsik (3) working with B. lactis aerogenes, and later Tomcsik and Kurotch-kin (4) employing B. lactis aerogenes, the pneumobacillus, and a yeast, isolated carbohydrate substances which produced anaphylactic shock in guinea pigs passively sensitized with homologous immune serum.   Lancefield (5) also obtained from streptococci carbohydrate material with which anaphylaxis could be induced in guinea pigs passively sensitized with anti-streptococcus serum.   Because of the presence of small amounts of nitrogen in the products, none of these authors felt justified in concluding that the carbohydrate alone was responsible for the shock.   Avery and Tillett (6) employing the highly purified polysaccharide of the type-specific pneumococci showed that guinea pigs passively sensitized with homologous anti-pneumococcus rabbit serum were thrown into anaphylactic shock by the subsequent injection of the homologous specific carbohydrate. Guinea pigs could not, however, be actively sensitized with the purified polysaccharides alone.   Since the materials used in those experiments were protein-free, and in the case of the Type II and Type III substances also nitrogen free, the results conclusively demonstrate the capacity of complex sugars to induce anaphylactic shock in animals passively sensitized with antibacterial sera.

The immunological specificity of pneumococcus polysaccharides has a close analogue in the serological specificity exhibited by gluco-protein and galacto-protein.   The immunologic behavior of the synthesized sugar-proteins led to their use in sensitization experiments. The results, reported in this paper, on anaphylaxis with artificially prepared carbohydrate-proteins confirm and extend the serological findings previously reported.   The production of both active and passive anaphylaxis was attempted in order to determine the sensitizing properties of the synthetic antigens and to demonstrate the specificity of the reactions.

Gluco-globulin represents phenol β-glucoside-azo-globulin.
Gluco-albumin represents phenol β-glucoside-azo-albumin.
Galacto-globulin represents phenol β-galactoside-azo-globulin.
Galacto-albumin represents phenol β-galactoside-azo-albumin.
The globulin was prepared from horse serum, and the albumin from egg white.

EXPERIMENTAL

Guinea pigs weighing 240 to 275 grams were employed. The sensitizing dose, whether serum or sugar-protein, was always injected intraperitoneally. The shocking dose was uniformly injected intravenously into a superficial vein of the hind leg.

For details concerning the chemical procedures involved in the synthesis of the materials, the reader is referred to the article by Goebel and Avery (1).

The serological characteristics of the serum employed and the method of preparation are described by Avery and Goebel (2).

Passive Sensitization

A. Results obtained with sugar-protein compounds

Eight guinea pigs were injected intraperitoneally with the pooled serum of three rabbits immunized with gluco-globulin. Five guinea pigs each received 5 cc. of serum, one received 3 cc., one received 1 cc., and one 0.5 cc. As previously mentioned, serum of this character possesses the capacity to precipitate complex antigens composed of heterologous protein conjugated with the homologous glucoside. The antigluco-globulin sera used in these experiments had an average titre of specific precipitins, as determined by tests made with gluco-egg-albumin, of 1 to 80,000. By reason of the fact that horse globulin alone when used as antigen does not elicit antibodies reactive with egg-albumin, the high precipitin titre of these sera is obviously dependent upon the conjugated glucoside radical.

Twenty-four hours after the administration of gluco-globulin antiserum, each pig received intravenously 1 cc. of gluco-albumin.

From Table I it can be seen that the five pigs sensitized with 5 cc. of serum all died with typical symptoms of anaphylactic shock.

The animals passively sensitized with 3 cc. and 1 cc., respectively, of antigluco-globulin serum had definite and typical symptoms immediately following the injection of 1 cc. of gluco-albumin but recovered. Pig No. 6, which received 0.5 cc. of serum, exhibited only a slight reaction.

A similar experiment was carried out using antigalacto-globulin serum for sensitization and galacto-albumin as the toxigenic antigen.

Sera obtained from three rabbits immunized with galacto-globulin were pooled; the precipitin titre, as determined with galacto-albumin, averaged 1 to 80,000. Eight guinea pigs were injected intraperitoneally as follows: 5 animals received 5 cc. of serum each, one received 3 cc., one received 1 cc., and one, 0.5 cc. The results given in Table II are equally as definite as those shown in Table I. All

TABLE I

Passive Anaphylaxis with Anti-Gluco-Globulin Serum[1]

Reactions induced by the use of the homologous glucoside conjugated with a heterologous protein—Gluco-Albumin

Guinea pig No.	Anti-gluco-globulin serum i.p.	Interval between injection of serum and shock dose	Shocking dose i.v.	Symptoms	Result
	cc.	*hrs.*	*gluco-egg-albumin*		
1	5	24	1 cc.	Typical	†3½ minutes
2	5	24	1 cc.	Typical	†3½ minutes
3	5	24	1 cc.	Typical	†2½ minutes
4	3	24	1 cc.	Marked scratching, bucking, coughing, respiratory distress	Definite symptoms followed by recovery
5	1	24	1 cc.	Violent typical symptoms	Definite symptoms followed by recovery
6	0.5	24	1 cc.	Occasional scratching	No reaction
			galacto-egg-albumin		
7	5	24	1 cc.	None	No reaction
			gluco-egg-albumin		
Same animal 4 hrs. later	—	—	1 cc.	Typical	†4 minutes
			galacto-egg-albumin		
8	5	24	1 cc.	None	No reaction
			gluco-egg-albumin		
Same animal 4 hrs. later	—	—	1 cc.	Typical	†4½ minutes

† Death of animal.
i.p. = intraperitoneal.
i.v. = intravenous.

pigs sensitized with homologous immune serum in amounts from 1 to 5 cc. reacted typically and fatally to the intravenous injection of 1 cc. of galacto-albumin; 0.5 cc. of serum was insufficient to sensitize.

TABLE II

Passive Anaphylaxis with Anti-Galacto-Globulin Serum

Reactions induced by the use of the homologous galactoside combined with a heterologous protein—Galacto-Albumin

Guinea pig No.	Anti-galacto-globulin serum i.p.	Interval between injection of serum and shock dose	Shocking dose i.v.	Symptoms	Result
	cc.	*hrs.*	*galacto-egg-albumin*		
1	5	24	1 cc.	Typical	†3 minutes
2	5	24	1 cc.	Typical	†8 minutes
3	5	24	1 cc.	Typical	†3½ minutes
4	3	24	1 cc.	Typical	†3 minutes
5	1	24	1 cc.	Typical	†3½ minutes
6	0.5	24	1 cc.	Slight scratching and coughing	Very mild reaction with recovery
			gluco-egg-albumin		
7	5	24	1 cc.	None	No reaction
			galacto-egg-albumin		
Same animal 1 hr. later	—	—	1 cc.	Typical	†3½ minutes
			gluco-egg-albumin		
8	5	24	1 cc.	None	No reaction
			galacto-egg-albumin		
Same animal 1 hr. later	—	—	1 cc.	Typical	†3 minutes

† Death of animal.

i.p. = intraperitoneal.

i.v. = intravenous.

In Tables I and II it is also shown that the reactions in guinea pigs induced with sugar-proteins and anti-sera are strictly specific.

Animals No. 7 and No. 8 of Table I received antigluco-globulin serum and 24 hours later were injected intravenously with galacto-albumin. No reaction occurred. Four hours later, the introduction of gluco-albumin induced typical fatal shock. Similarly pigs No. 7 and No. 8 of Table II, sensitized with anti-galacto-globulin serum, were unharmed by gluco-albumin; the subsequent administration of the homologous galacto-albumin antigen caused anaphylactic death of these animals. The animals also serve to demonstrate the fact that gluco-albumin and galacto-albumin are not primarily toxic.

B. Results obtained with uncombined glucosides

The specific inhibitory effect exerted by the glucosides on the precipitin reaction of sugar-protein and anti-sera has been described in detail by Avery and Goebel (2) and has been previously commented upon in this paper. It, therefore, seemed of interest to determine whether anaphylactic shock could be elicited by glucosides alone in passively sensitized guinea pigs, and if not, whether the inhibition which these substances have on the precipitin test would also be evident in the anaphylactic reaction.

As shown in Table III, two pigs (Nos. 1 and 2), sensitized 24 hours previously with antigluco-globulin serum, were injected intravenously with 1 cc. of the un-combined homologous glucoside. No reaction occurred. Two hours later 1 cc. of gluco-albumin injected into the same animal caused prompt anaphylactic death.

In guinea pigs Nos. 3, 4, and 5, the introduction of glucoside alone was followed immediately by an injection of gluco-albumin. Except for slight scratching, no response was elicited.

From these results it may be seen that the injection of glucoside into a sensitized animal exerts a definite but transitory protection against the shocking capacity of material which otherwise would be fatal. That the protective action of the glucoside is specific is demonstrated by guinea pigs Nos. 6, 7, and 8. These animals were injected with the heterologous galactoside; when, immediately thereafter they were given gluco-albumin no protection resulted and they died promptly with typical anaphylactic shock.

Table IV presents the results obtained with guinea pigs, which, after having been sensitized with antigalacto-globulin serum, were protected by the galactoside from the toxigenic effect of galacto-albumin.

Passive Anaphylaxis with Anti-Gluco-Globulin Serum

Effect of Uncombined Glucoside

Guinea Pig No.	Anti-gluco-globulin serum i.p.	Interval between injection of serum and anaphylactic test	Injection of glucoside i.v.	Result	Interval between injection of glucoside and sugar-protein	Injection of sugar-protein i.v.	Symptoms	Result
	cc.	*hrs.*	*glucoside*		*hrs.*	*gluco-egg-albumin*		
1	5	24	1 cc.	No reaction	2	1 cc.	Typical	†4 minutes.
2	5	24	1 cc.	No reaction	2	1 cc.	Typical	†4 minutes
3	5	24	1 cc.	No reaction	Followed immediately by	1 cc.	Scratches slightly, no other symptoms	No reaction
4	5	24	1 cc.	No reaction	Followed immediately by	1 cc.	Scratches slightly, no other symptoms	No reaction
5	5	24	1 cc.	No reaction	Followed immediately by	1 cc.	Scratches slightly, no other symptoms	No reaction
			galactoside					
6	5	24	1 cc.	No reaction	Followed immediately by	1 cc.	Typical	†7½ minutes
7	5	24	1 cc.	No reaction	Followed immediately by	1 cc.	Typical	†3¾ minutes
8	5	24	1 cc.	No reaction	Followed immediately by	1 cc.	Severe symptoms, falls on side. Apnoea; on feet	Severe shock followed by recovery

† Death of animal.
i.p. = intraperitoneal.
i.v. = intravenous.

TABLE IV

Passive Anaphylaxis with Anti-Galacto-Globulin Serum

Effect of Uncombined Galactoside

Guinea Pig No.	Anti-galacto-globulin serum i.p.	Interval between injection of serum and anaphylactic test	Injection of glucoside i.v.	Result	Interval between injection of glucoside and sugar-protein	Injection of sugar-protein i.v.	Symptoms	Result
	cc.	*hrs.*	*galactoside*		*hrs.*	*galacto-egg-albumin*		
1	5	24	1 cc.	No reaction	2	1 cc.	Marked scratching, coughing, backing, respiratory distress	Definite symptoms followed by recovery
2	5	24	1 cc.	No reaction	2½	1 cc.	Typical	†4 minutes
3	5	24	1 cc.	No reaction	2½	1 cc.	Typical	†3½ minutes
4	5	24	1 cc.	No reaction	Followed immediately by	1 cc.	None	No reaction
5	5	24	1 cc.	No reaction	Followed immediately by	1 cc.	None	No reaction
6	5	24	1 cc.	No reaction	Followed immediately by	1 cc.	None	No reaction
7	5	24	*glucoside* 1 cc.	No reaction	Followed immediately by	1 cc.	Typical	†2½ minutes
8	5	24	1 cc.	No reaction	Followed immediately by	1 cc.	Typical	†3 minutes

† Death of animal.
i.p. = intraperitoneal.
i.v. = intravenous.

TABLE V

Desensitization Induced in Passively Sensitized Guinea-Pigs by Injection of Glucoside and Homologous Sugar-Protein

Guinea Pig No.	Serum used for passive sensitization i.p.	Previous tests. Injections made 24 hrs. after serum i.v.	Result	Interval between previous tests and desensitization tests	Material injected i.v.	Symptoms	Result
				hrs.			
1	5 cc. anti-gluco-globulin serum	1 cc. glucoside followed immediately by 1 cc. gluco-egg-albumin	No reaction	4½	1 cc. gluco-egg-albumin	None	No reaction
2	5 cc. anti-gluco-globulin serum	1 cc. glucoside followed immediately by 1 cc. gluco-egg-albumin	No reaction	4½	1 cc. gluco-egg-albumin	None	No reaction
3	5 cc. anti-gluco-globulin serum	1 cc. glucoside followed immediately by 1 cc. gluco-egg-albumin	No reaction	4½	1 cc. gluco-egg-albumin	None	No reaction
4	5 cc. anti-galacto-globulin serum	1 cc. galactoside followed immediately by 1 cc. galacto-egg-albumin	No reaction	1	1 cc. galacto-egg-albumin	None	No reaction
5	5 cc. anti-galacto-globulin serum	1 cc. galactoside followed immediately by 1 cc. galacto-egg-albumin	No reaction	2	1 cc. galacto-egg-albumin	None	No reaction
6	5 cc. anti-galacto-globulin serum	1 cc. galactoside followed immediately by 1 cc. galacto-egg-albumin	No reaction	3½	1 cc. galacto-egg-albumin	None	No reaction

i.p. = intraperitoneal.
i.v. = intravenous.

The results are, in every respect, identical with those given in Table III both with regard to the transitory nature of the phenomenon and to its specificity.

The mechanism of the protection afforded by the glucosides is not as yet understood. The fact that the protective effect is no longer demonstrable after two hours indicates that "desensitization,"—if such has occurred—is transitory. Instances of what appears to be true desensitization have been observed in these experiments and are recorded in Table V.

Guinea pigs Nos. 1, 2, and 3, passively sensitized with antigluco-globulin serum, were protected from the shocking effect of 1 cc. of gluco-albumin by a previous injection of homologous glucoside. Four and one-half hours later the same pigs received a second injection of 1 cc. of gluco-albumin. No reaction occurred. The absence of shock following the second injection of whole antigen seems to be dependent upon the first dose of gluco-albumin. That the glucoside alone plays no direct part in the refractory state is demonstrated by its ineffectiveness when injected singly, two hours prior to the shocking dose (Table III). Pigs Nos. 4, 5, and 6 of Table V demonstrate the same principle, the difference being that antigalacto-globulin serum was used for sensitization, and galactoside and galacto-albumin were employed to complete the test.

C. Results obtained with uncombined protein

Avery and Goebel (2) have shown that the serum of rabbits immunized with synthetic sugar-proteins (gluco- or galacto-globulin) possesses two distinct antibodies; 1) the specific precipitin so intimately associated with the carbohydrate radical of the compound; 2) the "common" precipitin, reactive with globulin alone. Passive anaphylaxis experiments were, therefore, carried out, using pure horse globulin as the toxigenic material.

For sensitization, one pig received intraperitoneally 1 cc. of serum prepared by immunization with gluco-globulin; a second pig received 1 cc. of serum derived from a rabbit immunized with galacto-globulin. Twenty-four hours later each received 12 mgms. of globulin. Both animals died in typical anaphylactic shock (Table VI). A normal pig receiving the same dose of globulin gave no reaction.

TABLE VI

Passive Anaphylaxis with Anti-Gluco-Globulin and Anti-Galacto-Globulin Sera

Reactions induced by the use of horse globulin

Guinea pig No.	Sensitizing serum i.p.	Time interval	Shocking dose i.v.	Symptoms	Result
		hrs.			
1	1 cc. anti-gluco-globulin serum	24	1 cc. horse-globulin (12 mgms.)	Typical	†3 minutes
2	1 cc. anti-galacto-globulin serum	24	1 cc. horse-globulin	Typical	†2½ minutes
3	Normal control	—	1 cc. horse-globulin	None	No reaction

† Death of animal.

i.p. = intraperitoneal.

i.v. = intravenous.

Active Anaphylaxis

For purposes of testing the capacity of the synthetic sugar-proteins to produce active sensitization, 10 guinea pigs were injected intraperitoneally with 5 cc. of gluco-globulin and 10 other animals were similarly inoculated with 5 cc. of galacto-globulin.

In the preparations employed, 5 cc. of sugar-globulin contained 50 mgms. of protein. It is estimated (1) that 15 per cent by weight of this complex represents chemically combined glucoside. Consequently each pig received approximately 7.5 mgms. of the synthesized sugar-protein. The 20 pigs were tested 21 days later for active sensitization. As in the experiments on passive anaphylaxis, the sensitizing dose consisted of material in which the glucoside was joined to a protein heterologous to that used for sensitization. As previously mentioned, this precaution was taken in order to eliminate the possibility of protein-antiprotein reactions entering into the results.

In Table VII, the results of active sensitization obtained by the use of gluco-proteins are given.

Pigs Nos. 1, and 2, previously sensitized with gluco-globulin, were injected intravenously with 1 cc. of gluco-egg-albumin. Each promptly succumbed with typical symptoms. Pigs Nos. 3 and 4 of Table VII demonstrate the specificity of the sensitization; both of the animals when tested with 1 cc. of galacto-albumin showed no reaction. However, when, 3 hours later, 1 cc. of gluco-albumin was introduced, they reacted fatally. Pigs Nos. 5, 6, 7, and 8 were used to determine the influence of homologous uncombined glucoside on the reaction. In these tests the same relations were found to exist as described in the experiments on passive

TABLE VII

Active Anaphylaxis in Guinea Pigs Sensitized with Gluco-Globulin

Guinea Pig No.	Sensitizing dose i.p.	Interval between sensitizing injection and test	1st Injection Material i.v.	1st Injection Result	Interval between 1st and 2nd injection	2nd Injection Material i.v.	2nd Injection Result	Interval between 2nd and 3rd injection	3rd Injection Material i.v.	3rd Injection Result
	gluco-globulin	days		min.	hrs.		min.	hrs.		min.
1	5 cc.	21	gluco-egg-albumin 1 cc.	†5	—	—	—	—	—	—
2	5 cc.	21	1 cc.	†3½	—	—	—	—	—	—
3	5 cc.	21	galacto-egg-albumin 1 cc.	No reaction	3	gluco-egg-albumin 1 cc.	Severe symptoms. Recovery	—	—	—
4	5 cc.	21	1 cc.	No reaction	3	1 cc.		—	—	—
5	5 cc.	21	glucoside 1 cc.	No reaction	1½	1 cc.	†3	—	—	—
6	5 cc.	21	1 cc.	No reaction	2	1 cc.	†2½	—	—	—
7	5 cc.	21	1 cc.	No reaction	2	1 cc.	†4½	—	—	—
8	5 cc.	21	1 cc.	No reaction	Followed immediately	1 cc.	No reaction	—	—	—
9	5 cc.	21	galactoside 1 cc.	No reaction	4	galacto-egg-albumin 1 cc.	No reaction	½	gluco-egg-albumin 1 cc.	†4½

† Death of animal.
i.p. = intraperitoneal.
i.v. = intravenous.

TABLE VIII

Active Anaphylaxis in Guinea Pigs Sensitized with Galacto-Globulin

Guinea Pig No.	Sensitizing dose i.p.	Interval between sensitizing injection and test	1st Injection Material i.v.	1st Injection Result	Interval between 1st and 2nd injection	2nd Injection Material i.v.	2nd Injection Result	Interval between 2nd and 3rd injection	3rd Injection Material i.v.	3rd Injection Result
	galacto-globulin	*days*	*galacto-egg-albumin*	*min.*	*hrs.*		*min.*	*hrs.*		*min.*
1	5 cc.	21	1 cc.	†3½	—	—	—	—	—	—
2	5 cc.	21	1 cc.	†4	—	—	—	—	—	—
			gluco-egg-albumin			*galacto-egg-albumin*				
3	5 cc.	21	1 cc.	No reaction	2½	1 cc.	†4	—	—	—
4	5 cc.	21	1 cc.	No reaction	2	1 cc.	†3			
			galactoside							
5	5 cc.	21	1 cc.	No reaction	1½	1 cc.	†4½			
6	5 cc.	21	1 cc.	No reaction	3	1 cc.	†3			
7	5 cc.	21	1 cc.	No reaction	3	1 cc.	†2			
8	5 cc.	21	1 cc.	No reaction	Followed immediately by	1 cc.	Very slight symptoms. Recovery			
			glucoside			*gluco-egg-albumin*			*galacto-egg-albumin*	
9	5 cc.	21	1 cc.	No reaction	4	1 cc.	No reaction	1	1 cc.	†5
10	5 cc.	21	1 cc.	No reaction	4	1 cc.	No reaction	1	1 cc.	†3½

† Death of animal.
i.p. = intraperitoneal.
i.v. = intravenous.

anaphylaxis. When glucoside was injected immediately before gluco-albumin, complete inhibition of anaphylaxis resulted. However, when glucoside was injected one and one-half to two hours before the shocking dose (Pigs Nos. 5, 6, and 7), no protection occurred. Pig No. 9 of Table VII is further evidence of the specificity of active sensitization; in this animal attempts to inhibit shock with heterologous galactoside and to desensitize with galacto-albumin were ineffectual since the subsequent injection of gluco-albumin produced characteristic death.

TABLE IX

Active Anaphylaxis in Guinea Pigs Sensitized with Gluco-Globulin and Galacto-Globulin

Reactions induced by the use of horse globulin—the protein common to both antigens

Guinea pig No.	Sensitizing serum i.p.	Time interval	Shocking dose i.v.	Symptoms	Result
		days			
1	5 cc. gluco-globulin	21	1 cc. horse-globulin (18 mgms.)	Typical	†3½ minutes
2	5 cc. gluco-globulin	21	0.5 cc. horse-globulin (9 mgms.)	Typical	†14 minutes
3	5 cc. galacto-globulin	21	1 cc. horse-globulin (18 mgms.)	Typical	†3 minutes
4	5 cc. galacto-globulin	21	0.5 cc. horse-globulin (9 mgms.)	Typical	†7½ minutes
5	Normal control	—	1 cc. horse-globulin (18 mgms.)	None	No reaction

† Death of animal.
i.p. = intraperitoneal.
i.v. = intravenous.

In Table VIII, a similar group of experiments was carried out employing galacto-globulin for sensitization instead of gluco-globulin. Galacto-albumin was the toxigenic agent; galactoside was injected for inhibition tests. Results comparable in every respect to those recorded in Table VII were obtained. Consequently a detailed description need not be given.

Table IX presents the results obtained in guinea pigs actively sensitized with gluco-globulin (animals Nos. 1 and 2) or with galacto-globulin (Nos. 3 and 4) and subsequently injected with horse globulin.

All the animals gave typical reactions. Active sensitivity in these pigs was in all probability induced by the uncombined globulin present in the sensitizing material.

DISCUSSION

The experiments reported in this paper demonstrate the capacity of artificially prepared sugar-proteins to produce both active and passive anaphylaxis. The tests were devised and carried out in such a manner as to emphasize the significance of the carbohydrate radical. The fact that guinea pigs, passively sensitized with antigluco-globulin serum, or actively sensitized with gluco-globulin, can be subsequently shocked with gluco-albumin, demonstrates that the antigen-antibody specificity in these instances is directly dependent upon the carbohydrate fraction of the antigenic compounds.

The introduction of the sugar radical into the protein molecule endows the new complex with a sharply defined specific antigenicity. This fact is brought out by experiments in which galactoside was substituted for glucoside in the preparation of sugar-proteins used for sensitization. The same specific relations hold in the production of anaphylaxis with galacto-proteins as that described for gluco-proteins. Attempts to incite anaphylactic shock with heterologous material were ineffectual. The results of the anaphylactic experiments conform to the results anticipated by the serological findings of Avery and Goebel (2).

Landsteiner (7), employing complex antigens, has reported experiments on anaphylaxis of a similar character to those presented in this report. He found that guinea pigs sensitized with one azoprotein could be shocked by the injection of a second compound containing the same azo-groups attached to a different protein.

In addition to the new specificity which the carbohydrate radical confers upon the conjugated proteins, the uncombined glucosides by themselves also exert a definite influence on the reactivity of sensitized animals. When sensitized pigs are injected with the homologous glucoside immediately before the introduction of the toxigenic sugar-protein, they are completely protected from shock. However, the protection afforded by the glucoside alone is apparently only transitory; for, when the interval between introduction of glucoside and

shocking agent was as long as two hours, the injection of homologous sugar-protein produced prompt and typical anaphylactic death. That the temporary protection just mentioned is specific, was demonstrated by the experiments in which uncombined carbohydrate of heterologous type was shown to exert no such protective action.

The transitory, specific protection afforded by the glucosides alone is not yet understood. Landsteiner (7) found in experiments on anaphylaxis with azoproteins that the azo-component, when injected one hour before the conjugated azoprotein, inhibited shock. He considered that a state of anti-anaphylaxis had been induced. In the tests with glucosides and sugar-proteins, sufficient evidence has not been obtained to interpret the mechanism other than to say that the inhibitory effect of the glucosides disappears in at least two hours.

Active and passive anaphylaxis has also been elicited with uncombined globulin. Whether animals were passively sensitized with antigluco-globulin or antigalacto-globulin serum, the toxigenic action of globulin was equally effective. Since the sera employed contained anti-globulin antibodies, these results were to be expected. Guinea pigs actively sensitized with either gluco-globulin or galacto-globulin, were found to be equally sensitive to uncombined globulin. Since the material used to produce active sensitization contained free globulin, the subsequent intoxication with horse globulin is obviously based on a simple protein- anti-protein reaction.

CONCLUSIONS

1. Guinea pigs passively sensitized with the serum of rabbits immunized with an artificially prepared sugar-protein (gluco-globulin) exhibit typical anaphylactic shock when subsequently inoculated with gluco-albumin; the serum of rabbits immunized with a second synthetic sugar-protein (galacto-globulin) similarly sensitizes guinea pigs to galacto-albumin. The reactions, in each instance, are specific and depend for their specificity on the carbohydrate component, and not on the protein fraction of the synthesized sugar-protein.

2. Guinea pigs actively sensitized with gluco-globulin or galacto-globulin are similarly subject to anaphylactic shock, when injected, after 21 days, with sugar-proteins containing carbohydrate identical with that present in the sensitizing antigen, regardless of the kind of protein with which it is combined.

3. The unconjugated glucosides, although themselves not capable of inducing shock, inhibit the anaphylactic reaction when injected immediately prior to the introduction of the toxigenic sugar-protein. The protective action of the glucosides disappears within two hours after injection. In order to elicit the phenomenon, the carbohydrate must be the same as that combined in the sugar-protein complex.

4. Anaphylactic shock may be induced by uncombined globulin in guinea pigs passively sensitized with either antigluco-globulin serum or antigalacto-globulin serum; globulin is similarly effective in animals actively sensitized with gluco-globulin or galacto-globulin. The reactions elicited by globulin alone are dependent upon the common protein present in the antigens, and exhibit only species specificity.

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THE LIVER AS THE SOURCE OF FIBRINOGEN

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The present work embodies studies on the problem of fibrin regeneration in rabbits deprived of the entire liver (1).

In the past the formation of fibrinogen has been ascribed to various organs. Claude Bernard in 1848 (2), Lehmann (3), and Brown-Séquard (4) believed they had found the blood of the mesenteric veins rich in the substance and the content of the hepatic and renal veins relatively poor. Years later these findings seemed to be confirmed by the work of Dastre (5–8). As result the liver was credited with the function of fibrinogen destruction while certain other organs, particularly the intestine, were looked upon as its chief site of origin. By others, in the meantime, the lungs and skin (6), the bone marrow (9, 10), and leucocytes (11) have been thought to form it. The more important of these views will be considered further on in connection with our findings.

In 1905 Doyon (12, 13) and his associates found that extensive degenerative changes in the liver were accompanied by a fall in the blood fibrinogen content of dogs poisoned by chloroform and phosphorus. They suggested the liver as a probable source of fibrinogen. In the last quarter of a century this view has steadily gained acceptance. Nearly all workers on the question of the origin of fibrinogen are now agreed that the liver is its chief source. Whipple and Hurwitz (14) have determined a striking correspondence between the extent of liver damage and the decrease of blood fibrinogen in dogs poisoned by chloroform and phosphorus, and they have suggested an hepatic origin of this blood protein. Goodpasture (15), studying fibrinogen regeneration in dog's blood, agreed with this view in part but concluded that the intestines act as a controlling if unessential factor in fibrinogen formation. More will be said of this view further on.

It remained for Meek (16) to demonstrate clearly a rôle for the liver in fibrinogen formation. This author proved that the substance is regenerated in the Eck fistula dog. But if ligation of the hepatic artery was practiced in addition and the occlusion of the portal vein made close to the liver after formation of the Eck fistula, no regeneration whatever occurred. Indeed there followed a depletion of the amount already present in the blood.

In a series of studies on fibrin metabolism in the dog, under normal and pathological conditions, Foster and Whipple (17–20) have recently brought further

evidence to show that the liver is the chief if not the sole source of fibrinogen formation, a view strengthened by the work of Schultz, Nicholes and Schaefer (21).

From the foregoing it is clear that the studies of recent years have been converging upon the liver as the source of fibrinogen. But certain cogent objections have prevented a definite conclusion in the matter. Much of the evidence for a liver origin of fibrinogen has been obtained by injuring the organ with chloroform, phosphorus or carbon tetra chloride. To accept this evidence one must assume that the drugs act only upon the liver, not elsewhere in the body. Williamson and Mann's (22) observations upon hepatectomized animals have clearly shown that this is not the case.

It has seemed wise to us to study again the problem of fibrin regeneration in liverless animals not subjected to the effects of poisons and not suffering from circulatory obstruction to organs other than the liver (1).

Fibrinogen Regeneration in the Normal Rabbit
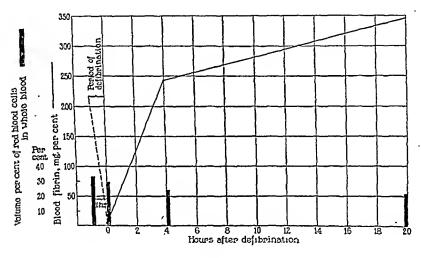
The span of life of the hepatectomized rabbit is short, on the average 24 to 30 hours (1, 23). Before proceeding to studies on the liverless animal it became necessary to estimate the rate and extent of fibrinogen regeneration during such a period in the normal animal.

Method.—Subtotal defibrination was carried out in the classical manner in four normal rabbits of about 2 kilograms each. Under ether anesthesia, cannulae were placed in the left carotid artery and jugular vein, and a sample of blood withdrawn for fibrin determination. A supply of blood roughly equivalent to the animal's estimated blood volume, already obtained from donor rabbits, was defibrinated after cross agglutination tests had ensured its compatibility with that of the experimental animal. The defibrinated blood was slowly injected through the cannula inserted in the jugular vein while at the same time an equal volume of arterial blood was removed from the carotid. This, defibrinated in turn, was reinjected into the animal during the removal of another equivalent volume of blood. Five or six repetitions of the procedure reduced the circulating fibrinogen to less than 5 per cent of its original amount. Blood specimens taken at intervals thereafter and examined for fibrinogen content disclosed the rate of its regeneration.

Using duplicate specimens, fibrinogen was estimated in the form of fibrin by the method of Foster and Whipple (17), as modified by Schultz, Nicholes and Schaefer (21). To ensure the complete precipitation of fibrin by the presence of ample thrombin, controls were run, as routine, to which fresh serum had been added. These controls showed no increased amounts of fibrin.

In normal rabbits fibrinogen regeneration was exceedingly rapid. Within five or six hours after a 90 per cent reduction of the substance

in the circulating blood a complete return to the previous amount was observed. Text-figs. 1 and 2 depict the findings in two of the four experiments. In the first one the blood fibrinogen had returned to the normal amount in less than 4 hours after total defibrination and 20 hours later had exceeded this by approximately 50 per cent. In the second instance a slightly less pronounced rise in blood fibrinogen during the first 5 hours was followed by a progressive increase to more than the normal amount in 21 hours and to double the original amount

TEXT-FIG. 1
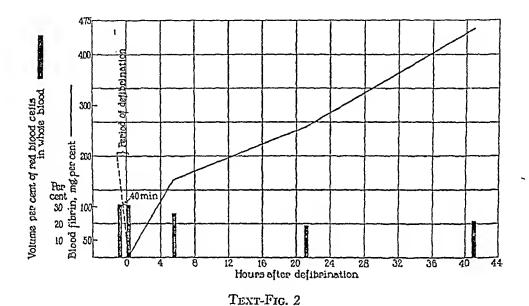
by the second day. The findings in the remaining two experiments were comparable to these in every way.

Influence of the Absence of the Liver on Fibrinogen Regeneration

The findings were wholly different in the liverless rabbit. Instead of a rapid fibrinogen regeneration, after partial defibrination, a speedy decrease in the small amount still remaining was the invariable rule.

Under ether anesthesia 10 rabbits of about 2 kilos body weight were subjected to a modified Markowitz operation (24) for inducing a collateral circulation about the liver, as already described by one of us (1). For two months thereafter they were kept on a full mixed diet on which they thrived, as evidenced by a gain in body weight. Then, after a fast of 48–72 hours, hepatectomy (1) was performed under ether and cannulae were placed in the left carotid artery and jugular vein.

In all the experiments blood specimens were drawn from the cannula placed in the carotid artery 10–15 minutes after hepatectomy. Partial defibrination of the circulating blood was next accomplished by the method described in the experiments on normal animals, save that it was not carried quite do far. The procedure accomplished a reduction of approximately 50–70 per cent of the circulating fibrinogen. Ten to fifteen minutes after completion of the partial defibrination another blood specimen was invariably taken. Further specimens were obtained at various time intervals in the different instances, as the charts

TEXT-FIG. 2
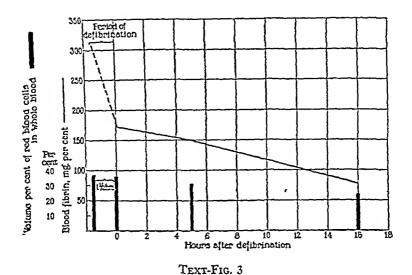
The Rapid Rate of Fibrinogen Regeneration in the Normal Rabbit

Text-figures 1 and 2 depict the rate of fibrinogen regeneration in the normal rabbit after almost complete defibrination of the circulating blood, as described in the text. It was exceedingly rapid. The line represents the concentration of fibrinogen in the blood after defibrination; the dark columns indicate the volume per cent of red blood cells in whole blood.
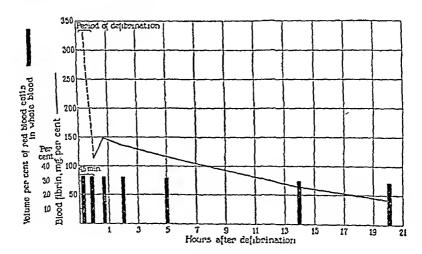
show, and the fibrin determinations carried out as in the preceding experiments. In the specimens taken after defibrination, controls were again run to which serum was added to make up for a possible thrombin lack. This addition did not appreciably increase the amount of fibrin clot obtained, showing that the fall in blood fibrinogen encountered was due to a lack of this protein and not to a fibrin ferment (thrombin) deficiency.

No evidences of fibrinogen regeneration appeared after hepatectomy in any of the experiments. Three typical examples, charted in

TEXT-FIG. 3

Failure of Fibrinogen Regeneration in the Hepatectomized Rabbit
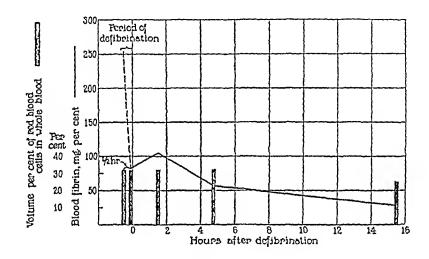
In Text-figures 3, 4 and 5 are charted the changes in blood fibrinogen of hepatectomized rabbits after partial defibrination of the circulating blood. The curves, which are fully discussed in the text, demonstrate the failure of fibrinogen regeneration in the liverless rabbit.

TEXT-FIG. 4

Text-figs. 3 to 5, show this fact well. Not only was there no rapid new formation of fibrinogen, such as occurs in the normal rabbit, but a swift fall in the amount of the substance in the blood signified a rapid utilization of the fibrinogen remaining in the organism.

The curves plotted in the text-figures differ in some details but the general findings are the same, and the variations can be safely attributed to the different time intervals at which blood specimens were taken.

TEXT-FIG. 5

Blood Fibrinogen after Partial Defibrination in the Liverless Rabbit

In the experiment plotted in Text-fig. 3, the fibrinogen content of the blood apparently decreased progressively, for a third specimen taken 5 hours after defibrination showed slightly less fibrin than the preceding one, and a fourth removed 16 hours later contained but half as much. In later experiments blood specimens taken at shorter intervals after defibrination showed a transient rise in the quantity in the blood, a phenomenon indicative of a fibrin reserve within the body as already noted by Foster and Whipple (20). The rapidity with which this occurred (Text-fig. 4) suggested an inflow of the substance with the lymph. In this experiment the third blood sample was obtained 35 minutes after the second, and a fourth an hour later. The fourth contained less fibrinogen than the third and each subsequent

blood specimen contained progressively less. Three similar experiments not plotted in the text-figures yielded like findings, no increase in fibrinogen being observed in the blood of hepatectomized rabbits later than 45 minutes after defibrination, that is to say after a transient rise. It is to be noted in Text-fig. 4 that the concentration of blood fibrinogen 5 hours after defibrination was still slightly above the figure obtained immediately following this procedure, that is to say in the second blood specimen. In Text-fig. 5 the plotted findings of

TABLE I

Blood Fibrinogen Content and Percentage of Red Blood Cells in Rabbit's Blood before and after Hepatectomy

No.	Preoperative		Postoperative		Hours after operation
	Fibrinogen, mgs. per cent	Volume per cent of red blood cells in whole blood	Fibrinogen, mgs. per cent	Volume per cent of red blood cells in whole blood	
1	380	33	130	31	30
2	408	32	156	34	27
3	447	36	222	33	27
4	345	34	201	33	24
5	262	30	115	32	16
6	426	30	282	28	15
7	310	35	171	34	15
Control Experiment. Ablation of 70 Per Cent of the Liver					
1	246	34	239	35	24

another experiment show the amount of fibrinogen in the fourth blood specimen, taken 5 hours after defibrination, to be distinctly less than in the second specimen which was removed a few minutes after defibrination. Such an experiment, without the determination shortly after hepatectomy, would have yielded a curve like that given in Text-fig. 3.

The investigation of the source of the fibrinogen reserve has not been attempted. Unfortunately fibrin determinations require so much plasma that frequent sampling is precluded and one is unable to follow at short intervals the changes in concentration of this substance in the blood of any one animal.

Blood Fibrinogen after Hepatectomy without Defibrination

In seven hepatectomized rabbits, which were employed for other experiments which involved no other complicating factors, the blood fibrinogen was estimated prior to removal of the liver and again 15–30 hours later. The results summarized in Table I are consistent. In the absence of the liver the fibrinogen concentration of the circulating blood rapidly decreased in all these animals while the hematocrit readings showed no significant changes. The findings are included here as further evidence not only of the lack of fibrinogen regeneration in the hepatectomized organism but of its destruction.

In a single experiment, also summarized in Table I, blood fibrinogen estimations were made before removal of 70 per cent of the liver of a rabbit and again 24 hours afterwards. As is well known, this procedure fails to induce clinical signs of liver insufficiency in the rabbit (25, 26). The blood fibrinogen showed no decrease.

DISCUSSION

It is certain from our results that the liver is absolutely essential to the maintenance of the normal quantity of fibrinogen in the blood. But is the liver the only source of fibrinogen? This question has not been answered by these experiments, for they do not preclude the possibility of an inconsiderable extra hepatic formation of fibrinogen, one wholly insufficient to make up for its destruction in the course of normal events. An enormous over production of fibrinogen took place in the normal animals stimulated to regeneration of the substance by defibrination of the blood. Surely a similar activity should have been observed in liverless rabbits after defibrination had there been any other important source of fibrinogen besides the liver. And any considerable compensation should have prevented the speedy decrease of the substance in the blood.

Theories of Extra-Hepatic Fibrin Formation

In the past many theories of extra hepatic fibrin formation have been offered. A little will be said of these.

The work of Mathews (11) and others (15) has tended to show an important activity of the intestinal tract in the origin of fibrinogen. At the present time it is evident that the findings of these authors were due to functional derangement

of the liver and not to injury or removal of the intestine. The experiments (11) had involved surgical interference with the blood supply of the intestinal tract, by ligations of the coeliac axis and of the superior and inferior mesenteric arteries. In addition ablation of segments of the intestine, or total extirpation of the organ, was practiced. For example, in one experiment (11) removal of the intestine from pylorus to rectum led to a pronounced fall in blood fibrinogen. In another experiment involving partial removal of the intestine in a cat, the stomach, pancreas, spleen and a segment of small intestine were allowed to remain. The animal, surviving 5 hours, regenerated fibrin but only half as rapidly as the normal cat. Procedures such as these reduced the circulation through the liver considerably.

The finding of fibrinogen in the blood of the mesenteric veins in greater concentration than in the general circulation has repeatedly been stressed in the literature as evidence for an intestinal origin of the protein (2, 3, 4, 11). The figures presented by the advocates of this view show changes sufficiently small to be accounted for by alterations in the concentration of the blood. The findings lie well within the errors of the methods used.

Müller (9) working with infections in guinea pigs found an increase in the fibrinogen content of the marrow in these animals and concluded that the marrow furnished this protein. In the light of more recent pathological knowledge these conclusions seem unwarranted. It is now well known from the work of Foster and Whipple and others (20, 11) that almost any infection or inflammation in the body leads to fibrinogen increase in the blood and accumulations of fibrin in various locations. Morawitz and Rehn (10) after withdrawing blood, defibrinating and reinjecting it into animals found myelocytic proliferation in the marrow and spleen and believed that this activity showed that fibrin deficiency was being made up in these regions. It is now recognized that red blood cells become fragmented and partially hemolyzed by the process of removal and defibrination. The myelocytic proliferation may have been a secondary consequence of this destruction.

One further point deserves mention. The experiments here described serve well to demonstrate the rapid rate of fibrinogen utilization, a phenomenon already emphasized by Foster and Whipple (19). It indicates an important function of the protein as yet unknown but worthy of investigation. As our experiments have indicated, absence of the liver brings about a true fibrinogen lack but no deficiency in thrombin (fibrin ferment), for an excess of the latter when added to the blood gave rise to no further coagulum of fibrin.

SUMMARY

In hepatectomized rabbits a progressive decrease in blood fibrinogen occurs. Partial defibrination in the liverless rabbit invariably

results in a progressive decrease in blood fibrinogen preceded by a temporary and slight rise. No evidence has been secured of fibrinogen regeneration in the absence of the liver. From this it follows that the liver is the essential source of fibrinogen and in all probability the sole one.

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STUDIES ON IMMUNOLOGICAL RELATIONSHIPS AMONG THE PNEUMOCOCCI

IV. ACTIVE IMMUNIZATION OF MICE AGAINST TYPE II PNEUMOCOCCI BY VACCINATION WITH YEAST

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Previous evidence (1) of immunological relationship between a variety of *Saccharomyces cerevisiae* and the Type II Pneumococcus consisted in the inter-reactions of the yeast and the bacteria in the antisera produced by immunization of rabbits with the two different microorganisms. This paper reports the active immunization of mice against Type II pneumococci by vaccination with yeast.

A group of mice were vaccinated with heated suspensions of yeast cells, and tested for active immunity by subsequent injection of virulent Type II Pneumococcus. Because of the known (2) irregularity in the active immunity responses of individual mice to homologous vaccination with Type II pneumococci, it seemed best to base the immunity tests of the yeast-vaccinated mice upon the percentage of animals surviving the test injection of a uniform dose of the Type II bacteria. In order to show that the protection afforded by vaccination with the yeast was not non-specific, groups of yeast-vaccinated mice were tested against comparable doses of Types I and III, as well as against Type II pneumococci.

EXPERIMENTAL

Methods: A group of 100 white mice of approximately the same age and weight were injected subcutaneously at 2 day intervals with 0.5 cc. of a 0.1 per cent. suspension of the dried yeast cells (boiled 5 minutes); after 12 subcutaneous injections and a rest of 3 days, two intraperitoneal injections were given. The active immunity tests were made on the 92 surviving mice 12 days after the last immunization injection.

The yeast-vaccinated animals were divided into three groups: 57 to be tested against Type II pneumococci, 20 against Type I, and 15 against Type III pneumococci.   A uniform dosage of 100 times the minimum required for death of normal mice was employed for the tests with the three types of pneumococci. The quantitative sufficiency of the test doses was controlled by injecting groups of normal mice with each culture.   The test strains were highly virulent for mice; the cultures used were inoculated directly from the heart's blood of mice.   The results are presented in Table I.

The results (Table I) showed that 20 of 57, or 34 per cent of the mice vaccinated with yeast were protected against subsequent infection with 100 lethal doses of virulent Type II pneumococci.   The uniform deaths in the control group of non-vaccinated mice proved that the infection test dose was sufficient to eliminate any irregularities

TABLE I

Specificity of the Protection of Mice against Type II Pneumococcus by Vaccination with Yeast

Tests against different types of pneumococcus	Vaccinated mice			Normal mice		
	Number of animals tested	Number survived	Percentage of animals protected	Number of animals tested	Number survived	Percentage of animals survived
			per cent			*per cent*
Type II	57	20	34	16	0	0
Type I	20	0	0	10	0	0
Type III	15	0	0	7	0	0

due to the possible occurrence of individual mice possessing unusual natural immunity against the Type II bacteria.   Hence, the vaccinated mice that survived can be considered to have acquired their immunity against Type II pneumococci by virtue of their vaccination with yeast.   Active immunity against Type II pneumococci is not easily obtained by mice even when vaccinated with the pneumococci themselves; and it is interesting to note that the percentage of individual mice that obtained anti-Type II immunity in response to the yeast vaccination is about the same as the percentage of mice that obtain immunity in response to homologous vaccination with the Type II bacteria (2).   The uniform death of the yeast-vaccinated mice tested against Types I and III pneumococci is important evi-

dence that the protection conferred by the yeast vaccination was specific to the Type II Pneumococcus.

COMMENT
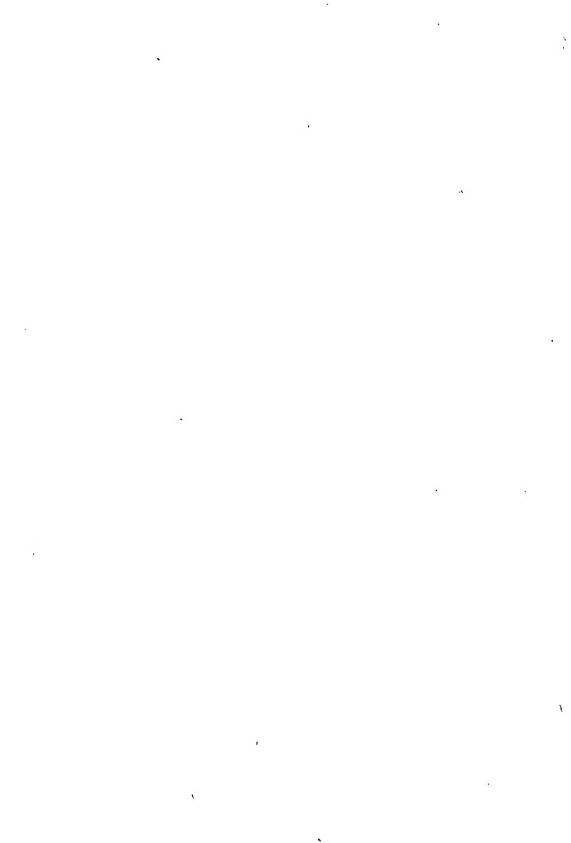
The described protection of mice indicates that these animals respond to yeast antigen with production of antibodies specifically reactive with Type II pneumococci. It had been shown (1) that rabbits can respond to the same yeast antigen with production of antibodies that agglutinate Type II bacterial cells, precipitate Type II culture filtrates, and confer passive protection against Type II infection; the more responsive rabbits yielding sera comparable in anti-Type II potency to the antisera produced by immunization with the Type II bacterial cells themselves. In another study (3) it was found that the relative antigenic effectiveness of two related strains of pneumococci differed in different species of animals. The present report therefore, is of interest as evidence that the immunological relationship between the yeast and Type II pneumococci causes mice, as well as rabbits, to respond to yeast antigen with production of antibodies specifically reactive with the Type II variety of Pneumococcus.

SUMMARY

Mice vaccinated with yeast were protected against subsequent infection with Type II pneumococci, but not against Types I or III. While the protection was not universal, as high a percentage of mice acquired active anti-Type II immunity, as was obtained by vaccination of another group of mice with Type II pneumococci themselves. This specific protection of mice by active immunization with the yeast antigen, is probably due to the same immunological relationship responsible for the anti-Type II reactivity of the antisera of rabbits immunized with yeast antigen.

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INFECTIVITY OF BLOOD DURING THE COURSE OF EXPERIMENTAL YELLOW FEVER

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A problem of importance in the study and control of yellow fever
is the period during which a patient may be a source of infection
through the bites of mosquitoes.

Work on this question was initiated by members of the American Commission
in Cuba (Reed, Carroll, Agramonte and Lazear (1)) who carried the disease from
person to person by *Aedes aegypti* which originally became engorged on the first
to third days of a patient's illness. Similarly, the French Commission in Brazil
(Marchoux, Salimbeni and Simond (2) and Marchoux and Simond (3)) found
that yellow fever could be transmitted to volunteers by mosquitoes fed on the
first, second and third days of disease and by blood or serum from a patient in
the same period. More recently, several workers have been able to study the
question of blood infectivity in human cases by the use of *Macacus rhesus* as the
experimental host. Bauer and Hudson (4) report that a lot of *A. aegypti* fed on
a human case 12 hours after onset of illness transmitted yellow fever to monkeys,
while a lot fed 36 hours after onset and the same period before death failed to
transmit it, although blood coincidentally withdrawn and injected into another
monkey induced a fatal attack. Mathis, Sellards and Laigret (5) infected
monkeys by means of the bites of mosquitoes which had been fed upon a patient
at the end of the first day of acute illness, and also by the injection of blood
withdrawn at the same time. Aragão (6) records the results of injecting monkeys
with patients' blood during the recent epidemic in Rio de Janeiro. He was
able to infect animals fatally with blood procured at various times up to 72 hours
after the beginning of illness, but not later; nor could he transmit the disease
by the inoculation of blood and organ emulsions obtained at necropsy.

The foregoing observations indicate a general agreement on the
infectivity of blood in human cases during the first three days of
illness. Whether the virus is in the circulating blood before the onset
of fever has been, of necessity, less readily determined. Marchoux
and Simond (3) report that in human beings their attempts to transmit

the virus by means of mosquitoes fed during the incubation period of yellow fever were unsuccessful. Kuczynski (7) has recently stated that the virus does not circulate in the blood during the incubation period, either in man or monkey.

The cardinal features of the human disease are reproduced in *Macacus rhesus*. With the purpose of throwing further light on the nature of the disease and the behavior of the virus, we have attempted to repeat and enlarge upon the experiments just referred to. Other

TABLE I

The Temperature Record of Monkeys after Infecting

Days after inoc-ulation	Monkey A		Monkey B		Monkey C		Monkey D		Monkey E	
	A.M.	P.M.	A.M.	P.M.	A.M.	P.M.	A.M.	P.M.	A.M.	P.M.
1	103.5	103.5	102.8	101.8	102.8	102.8	102.1	101.9	103.1	103.1
2	105.5	105.5	102.2	102.2	102.0	102.6	102.3	103.1	102.2	102.5
3	104.9	105.9	102.0	105.1	102.0	103.6	104.3	103.8 Dead	104.1	104.2
4	101.6	103.9	102.9	105.4	103.5	103.6			104.2	104.3
5	99.2	103.8	98.9	101.8	101.9	103.8			99.8	102.9 Dead
6	103.1	104.9 Dead	102.0	102.2	102.5	103.3				
7			103.7	104.8	102.4	103.7				
8			102.0	104.5 Dead	104.9	105.1				
9					105.4	105.1				
10					96.9	95.0 Dead				

advantages to be derived from these experiments are in connection with such laboratory procedures as the transmission of disease, maintenance of strains and, possibly, the preparation of "vaccines."

Five monkeys were infected with the Asibi strain of yellow fever, three by the bites of *A. aegypti* and two by the injection of monkey blood-virus. Attempts were made to transmit the virus from these animals at 24-hour intervals from the time of inoculation until death, by exposing them to batches of *A. aegypti* and by the injection of blood directly into other monkeys. In two instances, the same procedures were followed at the end of the first 12 hours as well. After appro-

priate periods, the mosquitoes were allowed to bite normal monkeys to test their infectivity.

The results are summarized in charts which plot also the highest daily temperatures of each animal; these readings, with only four exceptions, are the afternoon records. The morning and afternoon temperatures are tabulated in Table 1.

EXPERIMENTATION

M. rhesus A was injected with blood-virus, and on the second and third days after inoculation showed a marked febrile reaction. On the following two days, the temperature dropped. A terminal rise occurred on the day of death which was the sixth after inoculation. Each day and immediately postmortem, mosquitoes were fed on this animal and 0.5 cc. and 0.05 cc. blood were injected directly into other monkeys. The necropsy findings in A were typical of experimental yellow fever.

The temperature curve and results of attempts at transmission of disease are presented in Chart 1. This animal proved infective for mosquitoes fed from 24 to 96 hours after inoculation, but subsequent feedings gave negative results. Eleven of the twelve insects originally exposed at the first 24-hour interval failed to produce disease in a test animal 13 days after their original feed, but the same animal died when bitten 11 days later by 9 of these insects. A threatening epidemic in the next lot, fed at the febrile onset, necessitated exposure of the test animal on both the eleventh and fourteenth days after the original engorgement. It is interesting to note that one insect of this lot later caused fatal yellow fever by merely plunging its proboscis through the skin of a test *rhesus*, apparently without obtaining blood. Epidemics among the mosquitoes during the study of this and the following monkey necessitated varied and somewhat short periods of incubation in the insects to obtain a maximum longevity and number of bites. When the monkey was exposed to mosquitoes just postmortem, its peripheral circulation was so impaired that only one insect became engorged by feeding on a dependent part of the body.

Fatal attacks were caused by both doses of blood (0.5 cc. and 0.05 cc.) drawn on the first to fourth days, inclusive, after inoculation. Only the smaller amount on the fifth day induced fatal disease. On the day of death and at necropsy, the subinoculations were negative except for febrile reactions in three of the four monkeys.

M. rhesus B was infected by the bites of 9 *A. aegypti* and ran a typical course with death occurring on the eighth day. After 2 days of incubation, the animal registered 2 days of fever, followed by 2 days of low temperature and finally by the same period of thermal elevation. Each day and at the point of death, the monkey was exposed to separate batches of mosquitoes and 0.5 cc. and 0.05 cc.

blood were subinoculated directly into other animals.   The animal died of yellow fever, displaying the usual pathologic lesions.

Chart 2 outlines the results of attempts to transmit the disease, in relation to the temperature and course.   Mosquitoes fed on the second to fifth days transmitted yellow fever by bite to test animals.   The lots fed the days before and after the febrile period failed to induce infection after 13 and 10 days incubation,

	1	2	3	4	5	6	
						at 5 P.M.	at necropsy 10 P.M.
		Transmission by MOSQUITOES					
	13.. - 24.. +	11) 14) .. + 37... +	13.. + 35.. +	12.. + 35.. +	11.. - 24.. - 35.. -	10.. - 17.. - 34.. -	10.. - 20.. - 38.. -
106							
105							
104							
103							
102							
101		Transmission by BLOOD					
100	0.5cc + 0.05cc +	0.5cc + 0.05cc +	0.5cc + 0.05cc +	0.5cc + 0.05cc +	0.5cc F 0.05cc +	0.5cc - 0.05cc F	0.5cc F 0.05cc F
99							
98							

Injected with 0.5 cc. blood-virus

LEGEND: Data on mosquito transmission - figures refer to days between

original feed and subsequent test by engorgement.

+ = fatal yellow fever; F = fever and recovery; and - = no reaction.

CHART 1.   *M. rhesus* A

respectively, but killed the same test animals when fed at 25 and 20 days.   The two lots fed during fever transmitted the disease by bite at their first tests, 12 and 10 days after the original infective feed.

At the 24-hour interval after infecting, fatal results were obtained by the subinoculation of 0.5 cc. and not of 0.05 cc.   From the second to the seventh day, inclusive, both doses successfully transmitted the disease.   At necropsy, the smaller, and not the larger dose, was fatal.

M. rhesus C was bitten by 2 *A. aegypti* of an infective lot. The course of disease was quite different from the two preceding monkeys, and yet a course often seen in experimental yellow fever. After 2 days of incubation, the temperature rose to 103.6° to 103.8° on the third to fifth days, followed by a slight drop (103.3° and 103.7°) on the next 2 days. On the eighth and ninth days, the monkey registered temperatures above 105° and on the tenth went into collapse and died. *A. aegypti* were fed and subinoculations were made daily, as

	1	2	3	4	5	6	7	8 at 5 P.M.	at necropsy 10 P.M.
	Transmission by MOSQUITOES								
	12.. − 21.. − 44.. −	13.. − 25.. + 45.. +	12.. F 40.. +	10.. + 90.. F	10.. − 20.. +	9.. − 19.. −	9.. − 18.. − 36.. −	8.. − 18.. − 35.. −	8.. − 16.. − 39.. −
106									
105									
104									
103									
102									
101		Transmission by BLOOD							
100									
	0.5cc + 0.05cc −	0.5cc + 0.05cc +	0.5cc + 0.05cc +	0.5cc + 0.05cc +	0.5cc + 0.05cc +	0.5cc + 0.05cc +	0.5cc + 0.05cc +		0.5cc − 0.05cc +
99									
98									
	LEGEND: see CHART 1.								

(vertical axis label: Bitten by 2 *A. aegypti*)
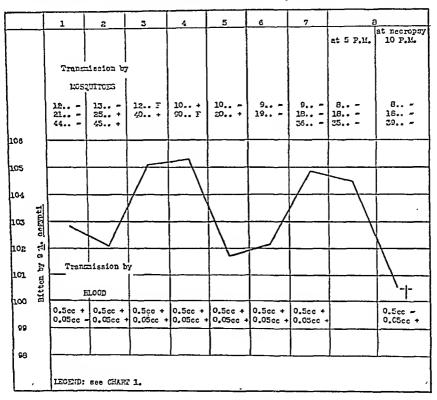
CHART 2. *M. rhesus* B

in the case of the previous monkeys, death occurring just after the last exposure to mosquitoes. The necropsy findings were typical of yellow fever in the monkey.

Chart 3 gives the daily temperature after infecting, as well as for the six preceding days, and the results of attempts at transmission. It is seen that the mosquito lots fed on the second to sixth days acquired infectivity. All of these lots were given a testing feed at 10 and 13 days after their original engorgement and if negative, subsequently at varying intervals. The third-day lot, only,

LEGEND: Data on mosquito transmission — figures refer to days between original feed on

M. rhesus C and subsequent tests by engorgement, E, or by injection, I.

+ = fatal yellow fever; F = fever and recovery; — = no reaction.
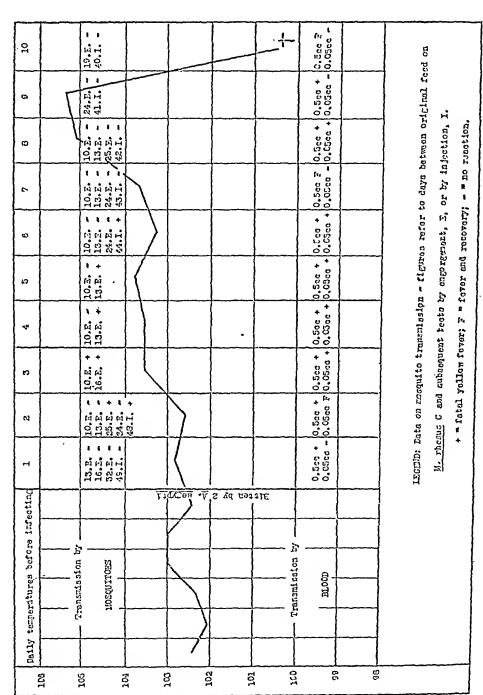
CHART 3. *M. rhesus C*

transmitted the disease at 10 days and the fourth and fifth at 13 days. Five, 10 and 6 insects of the 22 originally engorging on the second day, failed to infect test animals after 10, 13 and 34 days, but 10 of these insects induced fatal yellow fever 25 days after their infecting feed. The sixth-day lot failed to transmit by bite at 10, 13 and 24 days but later produced fatal disease when killed and injected, the number of specimens involved being 15, 9, 10 and 10, respectively.
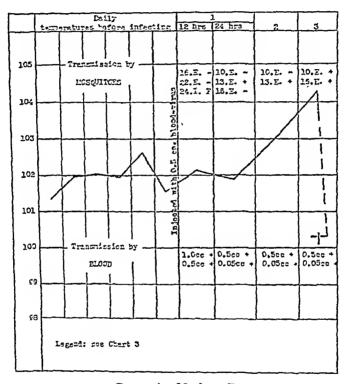
CHART 4. *M. rhesus* D

Only the larger subinoculated dose of blood (0.5 cc.) was fatal on the first 2 days after infecting. From the third to the sixth day both doses transmitted the disease fatally and on the seventh day, both amounts failed to kill. The two subinoculated animals died from the injections of the eighth day, and the monkey injected with the larger dose on the ninth day succumbed. Blood drawn at death was not fatal in either test animal.

M. rhesus D was infected by inoculation with monkey blood-virus. The course of the disease was fulminating, the animal dying with typical pathologic findings 82 hours after inoculation. Fever was registered only late in the course.

Transmissions were attempted with mosquitoes and blood at the end of 12 hours, as well as at the usual 24-hour periods.

Chart 4 presents the results in relation to the temperature record of the monkey studied. It will be noted that all lots of insects picked up the virus at each 24-hour feeding, the last being 10 hours before death. Transmission with the first lot was accomplished only by the injection of macerated insects; the injected monkey recovered, but a subinoculated animal died of yellow fever. By feeding 33 insects of the 24-hour batch after 13 days incubation, the disease was fatally

| Daily temperatures before infecting | | 1 | | 2 | 3 | 4 | 5 |
		12 hrs	24 hrs				
	Transmission by MOSQUITOES	No Lot Fed	13.E. — 22.E. — 24.I. —	10.E. + 17.E. +	10.E. + 13.E. +	10.E. — 13.E. +	10.E. + 13.E. +
	Transmission by BLOOD	1.0cc — 0.5cc —	0.5cc + 0.05cc —	0.5cc + 0.05cc +	0.5cc + 0.05cc F	0.5cc + 0.05cc —	0.5cc F 0.05cc +
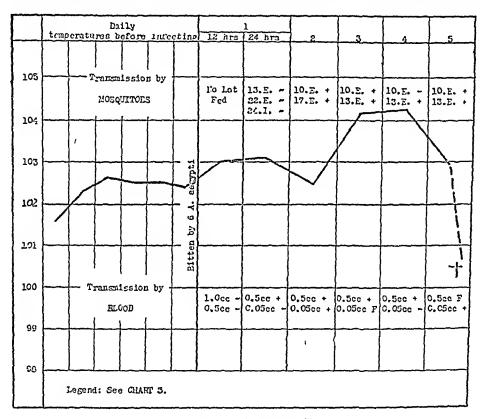
CHART 5.   *M. rhesus* E

transmitted, the 10 and 16-day tests, in which 34 and 31 mosquitoes, respectively, were used, being failures. The 48-hour lot induced infection at the 13-day test, but failed at 10 days, the latter animal later proving insusceptible to virulent material. The lots fed on this and the following monkey were manipulated so that by segregation of the insects engorging at the first test-feed, subsequent feeds by previously tested insects were insured.

All blood subinoculations were fatal, employing 1 cc. and 0.5 cc. doses at the end of the first 12 hours and 0.5 cc. and 0.05 cc. at the 24-hour intervals.

M. rhesus E was infected by the engorgement of 5 *A. aegypti* and displayed the usual course of experimental yellow fever and typical postmortem findings. , On the third and fourth days after infecting, the temperature was the highest (104.3°), preceded by a two-day incubation period and followed by a lowered temperature on the day of death. As with *M. rhesus* D, blood subinoculations were made at the end of the first 12 hours as well as at the 24-hour intervals; batches of mosquitoes were fed, however, only at the regular one-day periods, the last engorgement occurring 9 hours before death.

The results of the tests are recorded in Chart 5, together with the temperatures before and after infecting. All mosquito batches fed on this *rhesus* became infective with the exception of the first-day lot which failed to produce disease either by biting or injection. All tests after incubation periods of 10 or more days in the mosquitoes were successful, excepting the failure to produce disease by the fourth-day lot in an animal later shown to be insusceptible. The fifth-day batch became infected 9 hours before the death of the animal.

Neither dose of blood subinoculated at the end of the first 12 hours induced fatal yellow fever, and 24 hours after infecting, only the larger amount (0.5 cc.) was lethal. Blood drawn the second day transmitted fatal disease in 0.5 cc. and 0.05 cc. On the following 2 days, corresponding to the febrile period, the larger dose was effective. On the last day, 10 hours before death, 0.05 cc. transferred the disease fatally, the monkey that received the larger dose showing a delayed fever and recovering.

DISCUSSION

In order to interpret the results of these experiments, it is desirable to consider the course of experimental yellow fever in *M. rhesus*. The interval from the time of inoculation until death we have called the course of disease in the experimental rather than in the clinical sense. Clinically, it is difficult to compare the period of symptoms in the monkey with that in man. In the monkey, malaise and loss of appetite do not usually begin until a day or so before death. Vomiting rarely occurs and only when the animal is moribund. To determine the acute disease in *rhesus* monkeys, the urine findings and temperature records are alone available, but the examination of urine is not practical as a matter of routine. As regards temperature, various factors, such as a concurrent infection and excitement due to handling, may alter the reading. However, fairly reliable results are obtainable and records can usually be depended upon when the pre-inoculation temperature, the degree of apparent excitability and the presence of emaciation or diarrhea are taken into consideration.

For practical purposes, under the conditions existing in Nigeria, we have considered as fever a temperature of 104°F. or more, occurring after the expected period of incubation. Upon analysis of the records of 606 monkeys that died of yellow fever, only 3.8 per cent failed to reach this arbitrary limit and 1 per cent showed no registered rise, although it is possible that a brief thermal elevation may have occurred unrecorded. In considering an individual monkey, however, any appreciable rise may be significant.

In the application of these experimental results to the problem of yellow fever in man, the temperature records and the final outcome may profitably be compared. The five monkeys presented courses varying in length and with different thermal curves, but all were within the variations commonly observed in the experimental disease.

It is obvious that the multiplication of the virus in the body of the susceptible animal is extremely rapid, subinoculations of blood at the end of the first day regularly causing fatal disease in other monkeys, the same results being obtained at the end of the first 12 hours in the only blood-infected monkey tested at so short an interval. By the use of even a more delicate intermediary, the mosquito, the virus was transferred from mosquito-infected monkeys by bite at the end of the second day and from blood-infected animals as early as 24 hours after infecting. Moreover, high fever was induced in a test animal injected with insects fed on *M. rhesus* D 12 hours after its inoculation with blood-virus; although it recovered, a subinoculation during this fever resulted in fatal yellow fever in another *rhesus*. In considering these results in relation to the onset of fever, the virus proved by blood subinoculations to be in the circulating blood one and two days before fever. Of even greater interest is the fact that the virus was sufficiently highly concentrated for *A. aegypti* to become infective in every case on the day before the febrile onset.

In view of this early massive invasion of the animal host by virus, it is not surprising that the blood infectivity should continue through the febrile period. This was found to be true when infection was caused by mosquitoes as well as by blood injections. The two monkeys that received the smaller amounts of blood from *M. rhesus* E during its fever are the only recoveries from tests during this period; since mosquitoes coincidentally fed became infective, these failures

were probably due to incidental relative insusceptibility of the injected animals.

The thermal curves varied among the *rhesus* after the primary febrile period and are comparable to the variation seen among human cases at the corresponding interval. In the rapidly fatal course of *M. rhesus* D, the virus persisted in high concentration and was carried by blood and mosquitoes up to 10 hours before death, which was the final period of test. The same results were obtained from *M. rhesus* E, even though death occurred 36 hours after the last registered fever, except that only the final smaller dose of blood (0.05 cc.) was fatal. In the other instances, death occurred on the sixth, eighth and tenth days and was preceded by terminal rises in temperature. The mosquitoes did not become infective from these animals during the latter days and blood subinoculations were variable in result. It appears that in courses of longer duration, the virus tends to disappear from the circulating blood before death.

It should be noted that in the case of *M. rhesus* C, death of the animals did not result from the subinoculations of blood drawn the day before the terminal elevation in temperature, but on the two following days, blood caused fatal attacks in three monkeys. This is the only indication in these experiments of a possible reinvasion of the host by the virus coincident with a terminal fever. Of interest also is the fact that in three of the five monkeys on the last day of infectivity, only the smaller of the two doses used produced fatal infection. This occurred with material obtained in one case at necropsy, in another 9 hours before death and in a third instance 29 hours ante-mortem. Two explanations suggest themselves: either by chance the monkeys receiving the larger dose were relatively less susceptible, or at injection substances were transferred that protected the monkey or locally were inimical to the contained virus before the absorption of the larger amount of inoculum.[*]

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[*] Hindle in a paper that has just come to hand (*Trans. Royal Soc. Trop. Med. and Hyg.*, 1929, 22, 405) makes the suggestion that "—when large quantities of infective blood are used, the injection of the serum containing immune bodies at the same time as the virus may help the animal to recover from infection." We may also refer to two *rhesus* in these experiments injected with blood taken on the ninth day from the monkey that had been inoculated with

The results presented here from animal experimentation agree with those obtained by the study of human yellow fever in that the blood was found infectious and the virus transferable by mosquitoes for at least three days within the febrile period, except from *M. rhesus* D which died after the short interval of 82 hours after inoculation. They show also that infectivity for mosquitoes lasted longer than the initial rise in temperature and in two instances persisted to within a few . hours of death.

Of more importance is the fact, demonstrated by these experiments, that even before the onset of fever, the virus was of sufficient concentration to infect mosquitoes. *M. rhesus* has proved to duplicate the fundamental features (clinical, pathological and biochemical) of the human form of disease and there is reason to believe that the nature of the virus invasion is the same in man and monkey. If this is true, the results here obtained point to a better understanding of the condition in man and indicate a rational basis of study of some phases of human yellow fever. A close inquiry into the movements of a human case before onset of illness is suggested in order that his quarters might be freed of mosquitoes and the persons exposed detected and possibly treated with convalescent serum as a prophylactic measure (Bauer and Hudson (4)). Such precautions could be further extended to the careful observation and screening of previously exposed individuals. West African natives have yellow fever in variable degrees of severity, from mild, almost unrecognizable attacks to less often severe and fatal illnesses. This being the case, malaise and prostration are less commonly met than in Western Hemisphere subjects and an African native may be up and about while having a fever. Applying to these conditions the evidence furnished by the experiments reported here, the African patient is probably a source of infection for mosquitoes for one or more days before coming under the observation of those in charge of control work.

It has been hoped that immune serum would be effective therapeutically in human yellow fever. If this proves not to be the case,

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the first lot of mosquitoes fed on *M. rhesus* D (Chart 4). After exhibiting prolonged incubation periods, the animal that had received 0.2 cc. blood succumbed to yellow fever, while the other injected with 1 cc. had a febrile attack and recovered.

the explanation may be found in the fact that the virus appears to multiply with great rapidity, and invasion is early complete in the blood and perhaps in the tissues as well. Furthermore, in the study of material from monkeys killed on the first day of fever, we have regularly found, as has been reported by Aragão (6), that there is tissue damage in the form of marked fatty degeneration of the liver and sometimes of the heart musculature and beginning necrobiosis of hepatic parenchymal cells. Occasionally, also, albumin and casts are present in urine on the first day of fever, in the monkey as well as in man.

It has long been supposed that human necropsy material is non-infectious and we have met no record of fatalities attributable to postmortem examinations of yellow fever cases. Aragão (6) obtained negative results by injection of monkeys with necropsy blood and tissue emulsions. These observations point to a difference in the persistence of the virus in man and monkey since *rhesus* postmortem · blood and tissues are often infectious.

By these tests we have attempted to gain information on the nature and activity of the virus. The fact that the blood was fatally infectious as early as 12 hours after infecting up to death indicates simple multiplication of blood-virus rather than phases of development in the sense of metamorphosis. On the other hand, it does not explain the period of incubation necessary for the mosquito to become infective, whether by a cycle of development, multiplication, or penetration to the cephalic region. Bauer and Hudson (8) have shown by the injection of macerated mosquitoes that the virus is in an infective state throughout the incubation period in the insect. If, as seems unlikely, there is a change in morphology of either the mosquito-virus or blood-virus, each stage is infectious and the pathology and course of disease induced are the same as obtained under the usual conditions of virus transfer.

In connection with mosquito transmission, some features seem worthy of particular mention. Test feedings of lots of mosquitoes originally fed on monkeys A and B were made after varying periods of incubation, in several cases owing to rapid enfeeblement among the small number of insects. Particularly in tests of lots from *M. rhesus* B were there suggestions that the shortness of the incubation period

might be roughly proportional to the virus concentration in the donor animal. Those insects fed during the 2 days of fever transmitted the virus after 12 and 10 days, respectively, whereas the mosquitoes fed the days before and after fever failed at 13 and 10 days; and the latter lots tested on the same monkeys later induced fatal yellow fever. This idea seemed corroborated when studied systematically throughout the infective period of the course in *M. rhesus* C by test-feeding after 10 and 13 days incubation. The only lot transmitting at the shorter period was that fed at the initiation of fever on the third day after infecting the donor animal. The reasons for the failures of the infective lots originally fed on the second and sixth days are not apparent but it seems probable that only a small proportion of the mosquitoes were able to acquire an infection because of the low concentration of the virus at these times.

The rapidity of the courses in monkeys D and E did not allow for much illumination on these points. While insects fed at the height of registered fever in the former were the only ones transmitting the disease at 10 days, all but one of the infective lots fed on *M. rhesus* E transmitted at this period. It is remarkable that all infective lots fed on this animal, with the one exception, fatally transmitted the disease after 10 days irrespective of the time of original feeding. It is possible that feeding these lots after even shorter periods might have shown the same difference in rapidity to become infective as demonstrated in the previous experiments, especially since the mean atmospheric temperature (85.3°F.) was 10 degrees higher during the incubation periods than that for the first two series and 5 degrees above the mean during the incubations of the lots fed on *M. rhesus* C.

The failures of transmission by lots fed on the fourth day on monkeys C and E at the 10-day tests (see Charts 3 and 5), and on the first and second days on *M. rhesus* D at the 10 and 16-day tests, respectively (see Chart 4), are explained by the insusceptibility of the tested monkeys, as shown by later resistance to known virulent material. It should be noted here that all tests in these laboratories to date, using infected *A. aegypti* after 9, 10 and 11, as well as the accepted 12 days of incubation, have been positive in a total of 20 susceptible *rhesus* when the insects were originally fed at least during the febrile period.

The scope of the present study could hardly include more than a brief series of tests regarding the relation of the incubation in the insect host to the virus content of the donor animals. Evidence in three of the five groups of mosquito lots studied appears to indicate that when fed before or after the febrile period, there is not only a reduced proportion of infective mosquitoes of each lot, but an increased period required for the insects to become infective by bite. It is probable that the incubation periods in the insect host will be found to be influenced by temperature, but variations in transmission among the lots *of any one* series in the present experiments are not attributable to this factor. The greatest difference in the temperature means computed on a 2-hourly basis for the various lots was only 1.5 degrees.

Certain quantitative considerations in regard to the virus are brought out in several phases of these experiments. The animals injected with blood-virus showed a more rapidly fatal course here than did those infected by the bite of mosquitoes—a fact which has been observed in a large number of other tests. Similarly, blood subinoculations transferred the virus earlier and later in the course of disease, than did the insect host. The monkeys subinjected with blood died after varying intervals, but upon analysis it was found that in general the number of days before death depended upon the stage of the disease of the donor animal. The shortest average intervals (5.3 days) was shown by the 20 monkeys injected with blood of the febrile period, the next longer interval (6.5 days) by the 14 that received blood of the incubation period, and the longest (8 days) by the 16 inoculated with post-febrile blood. We have not found in other experiments that lethal doses of blood induce intervals before death depending in length on the size of the inoculum, except rarely when the minimal lethal dose is approached. When the result of the larger dose was compared with that of the smaller dose in these tests, the average intervals before death were the same, 6 days, in 27 and 23 monkeys in each group, respectively. We conclude, therefore, that quantitative factors did not control the time-result of injections of blood drawn during the periods of disease. It appears, rather, that the results depended on the "activity" of the virus or its ability to establish itself in the subinoculated animals, which was contingent on the stage of disease in which the virus was taken. The same considerations in

connection with mosquito transfers of virus did not reveal any relation between the number of mosquitoes 'or time of original feeding and the character of the course in the test animals.

These experiments suggest assistance in following certain laboratory procedures connected with the study of yellow fever. For transfer of virus, blood may be drawn with the expectation of a positive result over a wider period than for the transfer by mosquitoes. Small amounts are preferable to massive doses late in the disease. Mosquitoes appear to become infective by feeding throughout the febrile period. The proportion of an insect batch that picks up the virus at other times is probably low. We have not compared the titration of blood-virus and tissue-virus throughout the course of disease, but if the height of virus content of tissues corresponds with that of the blood, the optimum time for the preparation of tissue vaccine seems to be in the febrile period. If serum-virus is used for making vaccine, which has been found experimentally effective (Klotz and Hudson, unpublished), blood drawn during fever is undoubtedly desirable.

SUMMARY

Five *M. rhesus* fatally infected with yellow fever virus ran varied and typical courses, death occurring from 82 hours to 10 days after infecting. Batches of *A. aegypti* were fed daily on each monkey and specimens of blood injected into other animals. By mosquito transfer, the virus was found to be circulating in the peripheral blood 1 or 2 days after the infecting and the same interval before the onset of fever; in one instance, mosquitoes became infectious by feeding on a monkey 12 hours after its inoculation. Mosquitoes continued to · acquire infectivity during the febrile period and for 1 day thereafter, except in one instance .when death occurred during fever which prevented post-febrile testing. By subinoculation of blood, the disease was transferred before and after, as well as during the same interval as in mosquito transmission. In one of two attempts, the virus was carried by this means as early as 12 hours after the donor animal was infected. Following the first day of the post-febrile period, blood transmissions were irregularly fatal beyond the period infective for mosquitoes.
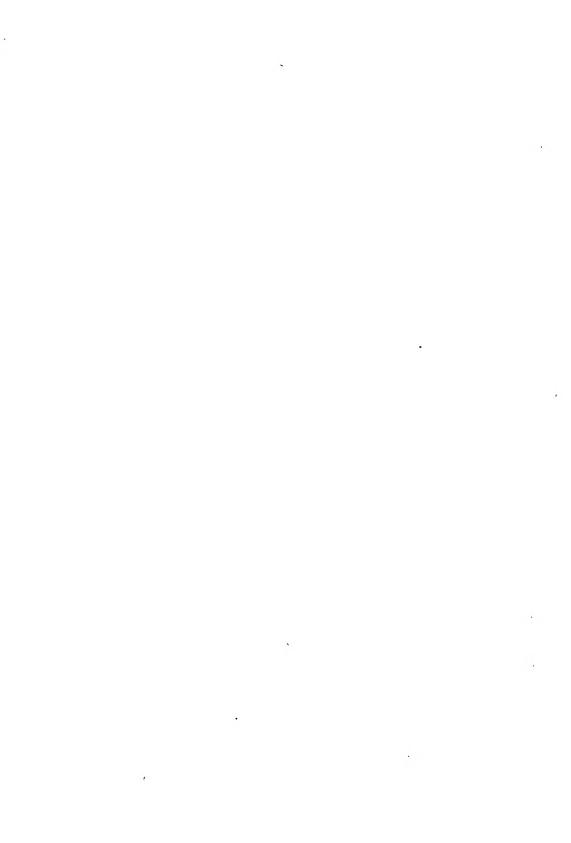
These results point to a remarkably rapid multiplication of the virus

in the animal host, in one case a blood subinoculation (0.5 cc.) being successful at the first test 24 hours after the donor monkey was bitten by only 2 *A. aegypti*. The regular acquisition of infectivity by mosquitoes fed during the incubation period is of especial interest in indicating the infectivity of human cases for mosquitoes before the appearance of clinical symptoms. This offers one explanation for the insidious propagation of epidemics of yellow fever and should be useful in the institution of control activities during an outbreak of this disease.

We wish to express our gratitude to Dr. Henry Beeuwkes, Director, for giving us helpful suggestions and for placing at our disposal all materials necessary for these experiments.

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A MECHANISM OF CONSERVATION IN THE KIDNEYS OF THE WINTER FROG*

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A well known peculiarity of the kidneys of "winter frogs" has been recently emphasized by Höber (1) in his studies of renal function by the method of perfusion. During the months of October, November, December, and January, he finds it often difficult to obtain a proper response by the kidney. This is most noticeable in the excretion of water, some kidneys forming no urine at all in spite of copious and long continued perfusion of both the glomerular and tubular circulations. The only explanation of this phenomonon we have been able to find that is based on experimental data, is that offered by De Haan and Bakker (2). They have observed that winter frogs show a definite decrease in their ability to excrete dyes. Fluorescene, a readily diffusible dye which is ordinarily excreted completely in one day, is not eliminated by the winter frog in several days. In the summer frog this substance is found in the urine in a concentration two or three times that of the blood, while in the winter animal the concentration in the urine is often less than that of the blood. Trypan blue, a less diffusible dye, is found in the urine only in a very dilute state. These observations are interpreted as being due to an increased density of the glomerular membrane which prevents their filtration and consequent appearance in the urine.

In the course of a study on experimental nephritis in frogs made by the method of perfusion, we have come in contact with this problem and as certain of our experiments would seem to offer another explanation of these peculiarities in the behavior of the winter kidney, they are here reported.

Methods

All of the experiments described were done during the months of December, January, and February. The frogs, R. catesbiana, averaging 850 grams in weight, were obtained from Louisiana. Here the animals hibernate intermittantly from about December 1st to April 1st, as during this period two or three light freezes and several frosts may occur. These cold spells were particularly severe during this last winter, (1928–1929) and it was on frogs collected in this period that our experiments were done. On arrival at the laboratory, the frogs were kept in large tanks of running water at a temperature of about 15°C. and were used within a week or ten days. Under these conditions they were quite active, and had all the appearance of summer frogs.

The method of perfusion which we have used is similar to that of Höber with the important modification of the perfusion pressures, our arterial pressure equaling 45 centimeters of water, while the venous pressure was 20 centimeters. The importance of this modification we have discussed in a previous paper, and will mention again in conclusion.

The perfusion fluid was the modified Locke's solution of Barkan, Broemser and Hahn (3) buffered to a pH of 7.4 and properly oxygenated. Glycocol to a concentration of .50 percent, and glucose to .05 percent were also added to the solution.

The details of the operative procedure have been previously described and will therefore not be repeated. Canulae from the perfusion flasks were placed in the anterior abdominal vein, thus supplying the renal-portal system, and into the aorta just below the junction of the two thoracic branches. Uretral cannulae were placed in the ureters, and the urine collected in measured periods of time. The samples of urine obtained were expressed as to volume in cubic centimeters per hour, the presence or absence of sugar tested with Benedict's Solution, the electrolyte content determined with a Christiansen ionometer, the reading being expressed as a percent concentration of NaCl, and any dye present determined quantitatively with a Dubosq colometer. All these determinations may be made with considerable accuracy, as the samples obtained are large, the normal volume of urine being from 8 to 10 centimeters per hour.

EXPERIMENTAL

Before detailing the results of perfusion of the kidneys of winter frogs, it is necessary to describe those obtained by similar procedures with the frog in summer when the organs of the animal are in a state of maximum activity.

Under these conditions, with the method described above the volume of urine from the two kidneys varies, as stated above, from 4 to 10 cubic centimeters per hour.

Sugar is normally absent from the urine, though it may be present in the first fifteen minute sample collected. Any persistent appearance of it indicates, as Höber has shown, a damaged kidney, and is usually accompanied by an abnormal increase in the amount of urine.

The electrolyte content of the urine under normal conditions is considerably lower than that of the perfusion fluid. The lowest figures which we have obtained are 20 percent of that of the Locke's solution, while as an upper limit, we have taken 50 percent. If it approaches nearer than this figure to the salt content of the perfusion fluid, sugar is usually present and the volume is large; in other words, there is evidence of what Höber has shown must be interpreted as tubular damage.

In a previous article (4) it was shown that the two dyes, neutral red and phenol red, are excreted in different manners. The former is eliminated almost entirely through the tubules, while the phenol red is excreted in by far the greater part through the glomeruli.

These two dyes were used in the perfusion fluid at a concentration of 12.5 mg. per 1000 cubic centimeters for neutral red, and 20 mg. per 1000 cubic centimeters for phenol red. The rate of excretion of phenol red by the normal kidney under these conditions varies from .5 to 1.2 mg. per hour. In spite of this variation in rate between the kidneys of different animals, abnormalities can be readily recognized as a rule by the inconstancy of the findings. An abnormal low excretion decreases progressively as the perfusion proceeds to a very low figure and is associated with other evidences of kidney damage, such as a high salt content, or the presence of sugar.

The concentration of the dye in the urine as compared to that of the perfusion fluid (Höber's "concentration factor") varies greatly, affected as it is by the volume of water excreted. A concentration factor as low as 200 percent may be found in urine which is otherwise normal, the rate of excretion of the dye remaining within normal limits due to a large volume of urine. With small volumes of urine as high a factor as 3000 percent may be obtained. On the other hand, when this figure falls as low as 100 percent, the urine contains sugar, a high sa't content, or some other evidences of kidney damage are present.

The rate of excretion of neutral red also varies considerably with different kidneys. From .45 mg. to 1.5 mg. per hour have been found under normal conditions, but here again the constance of a normal kidney is easily distinguished from the progressive fall observed with abnormal organs. The concentration factor, as in the case of phenol red, is dependent largely on the rate of water excretion. A factor as high as 7500 percent has been observed.

The Perfusion of Normal "Winter Frog" Kidneys

In the first five periods of Figure 1 is shown a typical experiment illustrating the effect of perfusion on the kidney of the winter frog.

The dye in the Locke's fluid was phenol red. It will be noticed that although in the first period the volume of water was within normal limits for summer conditions, 9 cubic centimeters per hour, during the first five periods there is a gradual and progressive decrease to 2.4 cubic centimeters per hour. The arterial perfusion flow was practically constant during this period, so that the decrease in volume can not be explained by any failure of the perfusion method. A faint trace of sugar was present in the first period, but disappeared in the next sample and remained absent during the remainder of the experiment. The salt content in the first period was 50 percent, decreasing to 45, and finally to 40 percent of the concentration in the perfusion fluid in period 5.

The phenol red, as in most of the experiments to follow, was introduced first into the tubular circulation, and then into the glomerular system. This was to test our previous findings, that the excretion of this dye is chiefly through the glomerulus. As is seen in periods 1 and 2, only a very low rate of excretion resulted, .02 and .04 mg. per hour. The rate rose however at once to .16 mg. per hour when it was led to the glomeruli, an eight fold increase. This rate is, however, still far below the usual figure for a summer frog, and in the following periods 4 and 5 it decreased progressively to a final figure of .07 mg. per hour. The highest concentration factor attained was only 115 percent.

The experiment described thus far, differs strikingly from the results of perfusion of a summer frog in that there was a gradual and progressive decrease in the excretion of all the elements of the urine. The water decreased, sugar disappeared, and salt became less, as did also the rate of excretion of phenol red. The concentration factor of the latter was, however, greater than 100 percent. In other experiments similar results were obtained, in some the volume of urine decreasing to less than .1 cubic centimeter per hour, and in other cases the volume from the beginning of the perfusion was so low that the perfusion might be said to be ineffective.

Viewing these findings in the light of De Haan and Bakker's theory that the winter frog's kidney is characterized by a decreased filtration of the urinary constituents through the relatively impermeable glomerular membrane, it is difficult to explain by such a mechanism

the concentration of the dye in the urine above that of the perfusion fluid. In other experiments, this concentration factor of phenol red rose to such figures as 410, 520, 540, 840, and even 1000 percent as the volume of water decreased. Obviously the concentration mechanism is still active in the winter frog's kidney and this fact led to the idea that perhaps all of its peculiarities might be explained by the assumption of an increased absorptive activity on part of its tubules.

The hypothesis is easily tested. The absorptive function of the tubules may be depressed, as Höber has shown by anesthesia with
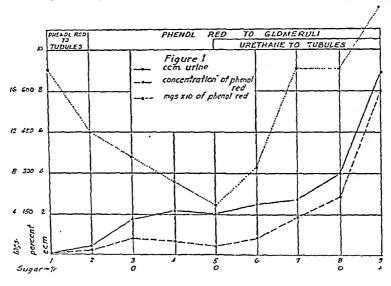
FIG. 1
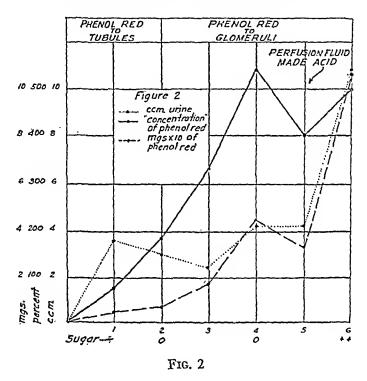
urethane, and if our assumption is true, such a procedure should cause the kidney of the winter frog to form a urine which is similar in amount and constituents to that of the summer frog. The second half of figure 1 (periods 6, 7, 8, and 9) shows such an experiment.

Beginning at the removal of sample 5, urethane in a concentration of 2 percent was added to the tubular perfusion fluid, and in periods 6 to 9 all the evidences of a depression of tubular function as described by Höber and observed by us in our previous article developed.

The rate of water excretion increased steadily until it reached 12 centimeters per hour, a figure which equals the output of the summer frog. An equally striking increase is noted in the output of phenol red. The rate of excretion was

doubled in the first period following the anesthesia and progressively increased until in the ninth period it reached a figure which equals even the maximum figure attained by summer frogs, that is, 1.6 mg. per hour.

That these increases in water and dye output are associated with a failure of tubular absorption, is seen by the changes in salt and sugar excretion. Absorption of salts remained active until the eighth period, when the concentration rose to 50 percent of that of the perfusion fluid concentration and finally to 60 percent in the last period, at which time a trace of sugar also appeared in the urine.

FIG. 2

These results are not the peculiar effect of the anesthetic, for any damage to the tubular epithelium which produces a depression of its function results in similar effects. In figure 2 the depression of tubular absorption was produced by frank damage to the epithelium with an unsuitable acid perfusion fluid. Under these conditions the scanty winter urine, poor in dye content, not only increased to values in water and dye which might be considered normal for a summer frog, but continued to show a decrease in tubular absorption until a point of frank abnormality was reached.

The perfusion was started with Locke's solution at the normal pH of 7.4 and the usual amount of phenol red. The volumes of urine in the first 5 periods were

low or moderate in amount, and contained no sugar. While the phenol red was administered to the tubules, (periods 1 and 2) the rate of excretion was very low, .08 mg. per hour, and increased over five fold to .44 mg. per hour, when it passed to the glomeruli (period 4). This figure is however lower than that of the average summer frog and begins to fall in the 5th period. 'At this time the perfusion fluid to the tubules was made acid to a pH of 5.9. The epithelial cells were immediately damaged, and their absorptive activity destroyed as evidenced by the appearance of sugar in the urine. As a result, the rate of water output rose to what might be considered a normal figure for the summer frog, 10.8 cubic centimeters per hour, and the rate of excretion of phenol red to the high rate of 1.6 mg. per hour.
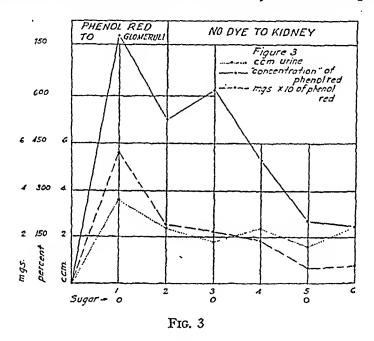
These experiments support strongly the idea that the apparent "inactivity" of the winter frog's kidney is the result of an increased activity on the part of its absorbing tubules rather than that there is any decrease in the process of glomerular filtration. Both water and dye are present in sufficient amount in the tubular lumen and are excreted readily when the tubule cells are prevented from absorbing them.

In considering the latter part of the two experiments just described, in which the excessive absorbing activity of the tubules has been depressed either by anesthesia or by frank damage to the tubule cells, one is struck by the fact that not only is a large amount of dye excreted, but also that although the normal concentrating mechanism, absorption of water, has been depressed, never the less the dye is still found in considerably higher concentration in the urine than in the perfusion fluid. Evidently then, some water is being absorbed from the glomerular filtrate or there is an added source of dye output in these periods, or both of these possibilities exist.

Although it is impossible to answer such an involved question in any completely satisfactory quantitative manner, it can at least be shown that the second named factor may well play an important part in the concentrating process. The following experiment (Fig. 3) shows that under certain conditions an added source and mechanism exists for the excretion of phenol red which may not function to any significant degree under normal conditions of renal activity.

Clear Locke's solution was supplied to the tubules while phenol red in the usual concentration was introduced into the glomerular circulation. The dye was excreted in high concentration, (780 percent) and at the moderately high rate of

.56 mg. per hour. As is typical of the winter frog, the next period showed a drop
to about one half this figure, .25 mg. per hour, the concentration factor was lower
and the volume of urine decreased. At the end of this period the dye containing
fluid of the glomerular circulation was replaced with clear Locke's solution, so that
no dye was being supplied to the kidney. Nevertheless, the rate of dye excretion
remained practically constant, .22 mg. per hour and since the volume of water
had continued to decrease, the concentration factor was somewhat higher, 620
percent. The following period also showed a high rate of excretion, but in the
5th and 6th periods the lack of dye supply began to make itself apparent, and the
rate of excretion and the concentration factor of the dye fell to a low figure.

FIG. 3

For one half hour, therefore, the kidneys had maintained the rate
of excretion and concentration of the dye without the usual source
of glomerular supply. Since no other tissues were included in the
perfusion system, it is obvious that the source of this excreted dye
must have been the kidney itself.

It has been shown by many observers that a storage and concen-
tration of phenol red occurs in the cells of the renal tubule (Marshall
and Crane (5)) and Hayman and Richards (6) have proved by direct
observation that when introduced into the capsular space of the
glomerulus the dye appears within the tubule cells. Richards and
Barnwell have furthermore shown (7) that although normal tubule
cells are able to maintain a higher concentration of phenol red than

exists in the fluid in which they are immersed, when damaged this is no longer the case. With these facts in mind, it seems clear that the dye which had been stored during the first periods of the experiments described was subsequently liberated from the tubule cells and excreted during the period of dye free perfusion. Such a source and method of dye excretion is particularly important in the winter frog, for the kidneys of such animals whose absorbing mechanisms are extraordinarily active, are especially rich in stored dye, so that when damaged by the urethane as in experiment 1, a considerable amount may be added by liberation from the tubule cells to that amount which is being eliminated by glomerular filtration.

Evidence has been presented which has been interpreted as indicating that the kidneys of winter frogs conserve water, salt, sugar, and, when present in the urine, dye, by a process of excessive absorption from the urine within the renal tubule. It has been shown by Scheminzky (8) and by the writers, that neutral red is excreted almost entirely through the tubule epithelium. Its manner of excretion is therefore different from that which we have found for the excretion of phenol red. In the kidneys of the winter frogs which are so active in conservation by their mechanism of absorption, one might expect a similarly conservative action on the part of the excretory function of the tubules. The following experiment shows this to be the case (Fig. 4).

Neutral red was introduced into the tubular circulation. In the first period it was excreted at a moderate rate (.45 mg. per hour) and in high concentration (735 percent), but in the next two periods (2 and 3) there was a marked fall in both rate of excretion and concentration. The final figure of .03 mg. per hour and a concentration factor of 100 percent are far below any figures obtained with normal summer frogs. The volume of urine decreased and the absorption of salt was active as its concentration in the urine was only 40 percent of that of the perfusion fluid in the 3rd period.

The kidneys of the winter frog therefore excrete neutral red to a lesser degree than those of the summer frog and the conservative mechanism of excessive absorption is complemented by a decrease in the excretory function of the tubular epithelium. The experiment was now continued for the examination of another point of interest.

It has been shown that phenol red which has been absorbed by the tubule cells from the lumen of the tubule may be subsequently excreted back into the lumen when the concentration of dye in the urine is low or when the tubule cells have lost their absorptive power as a result of damage.

The findings in the fourth and fifth periods show that there may be an analogous excretion of stored neutral red from the tubule cells. At the end of period 3 the perfusion fluid containing neutral red was replaced by dye free Locke's solution. The kidney was now receiving no dye, yet during the next period the rate of elimination of neutral red remained nearly as high as the preceding period and the urine was more concentrated in dye than the perfusion fluid. By the sixth period both rate of excretion and concentration factor had fallen, evidently due to the exhaustion of the available neutral red which had been stored in tubule cells.[1]

It will be noticed in the sixth period that the volume of urine was small, 1.2 centimeters per hour, and the salt concentration was especially low, only 30 percent of the concentration of salt in the perfusion fluid. This is strong presumptive evidence that we are dealing with typical winter kidneys, that is, that their absorptive mechanisms are very active. Nevertheless, this point was tested with phenol red as in our previous experiments. This dye was supplied to the glomeruli during the 7th period and in the 8th period was excreted at a moderate rate, .24 mg. per hour and in moderately high concentration, 495 percent. At the beginning of the 9th period tubular absorption was depressed by administration of urethane in a concentration of 2 percent to the tubules. The rate of water elimination immediately rose to 10.6 centimeters per hour and there was an increase in the rate of phenol red excretion to .66 mg. per hour. The absorptive mechanism of the tubules had indeed been so depressed by the anesthetic that the concentration of salt rose to 60 percent of that of the perfusion fluid and sugar appeared in the urine.

In the same winter kidneys, therefore, an excessive absorptive activity and a decreased excretory function was demonstrated in the tubular epithelium.

DISCUSSION

From the experiments described above, the conclusion has been drawn that in winter frogs there exists a profound alteration in the function of the tubular apparatus of the kidneys. This alteration is manifested by an increase in the activity of their resorptive processes and a decrease in their excretory functions. The method of demon-

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[1] Scheminzky has described the storage of neutral red in the tubular epithelium and we shall later discuss this point more fully in a study of experimental nephritis by means of vital staining.

stration of these facts included the study of substances such as water, salts and sugar, which are normally present in the blood and urine, but the most striking demonstration was made with the dye, phenol red.

In a previous article (4) we have shown why we believe that the principal method of phenol red excretion is through the glomerulus.

While this article was in press, there appeared a study by Scheminzky, working in Höber's laboratory, who from somewhat similar experiments came to a different conclusion. He found, as did we, that neutral red was excreted by the tubules and only slightly by the glomerulus, but contrary to our conclusions, he believes that phenol red is also excreted principally by the tubules.

It is impossible to adequately discuss this important and lengthy paper, and we shall only attempt to call the reader's attention to certain points which we feel should be especially noted in comparing the results of the two investigations.

In the first place the type of experimental animal is different. Scheminzky used winter animals, and such material judged in the light of De Haan and our present paper is obviously not the most satisfactory for the study of the method of normal dye excretion. As a result, the volumes of urine, and amounts of dye excreted are very small. In one emphasized experiment .19 cubic centimeters per kidney per hour is given as a normal volume and in another .86 cubic centimeters is cited as an example of diuresis due to tubular narcosis. The technical difficulties of quantitative NaCl and phenol red and qualitative sugar determinations all on a thirty minute sample under these conditions are manifest, especially when dye output must be expressed in millionths of grams.

Of even greater importance, however, is the difference in perfusion technique in Scheminzky's and our study. In most of his experiments he used a pressure of 18 and 9 centimeters of water for the arterial and venous systems and under these conditions the venous flow was greater than the arterial.[2] In our previous article

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[2] In discussing the improbability that fluid from the venous circulation has reached the glomeruli Scheminzky on page 667 gives a theoretical calculation as evidence of the impossibility of such an occurrence. Without going into the details which he presents we wish to point out that although it is correct that at least 9.1 centimeters of dye containing perfusion fluid must be assumed to have reached the glomeruli from the venous circulation in his hypothetical case, it is not at all necessary to assume that this was mixed in the glomeruli with the 40 cubic centimeters of dye free fluid which flowed through the aortic circulation. The flow through the glomerular capillaries may in fact have been purely venous and

we havè pointed out that in order to cause the kidney to function in a normal manner pressures of 45 and 20 centimeters are required. Detailed experiments have shown that such pressures produce no abnormalities in the formation of the urine by either the glomeruli or tubules and that only with them will perfused kidneys secrete a volume of urine which is comparable with what Adolph (9) has shown to be the normal rate of water excretion.[3]

As a result of these seasonal and technical differences, and also because our frogs were perhaps much larger, our volumes of urine are larger and may be considered as normal in regard to water excretion. The determinations of its constituents are made with ease and the results of dye excretion are expressed as milligrams.

So much for general differences in method. In the specific experiments certain differences should also be noted. In contrasting the excretion of phenol red by the tubules and by the glomeruli Scheminzky used different animals to test each part of the renal unit. The dye was found in the urine in concentrations higher than in the perfusion fluid after both tubular and glomerular administration and these results on different animals were then compared by averages and by "specific concentration" and "Leistungs" factors rather than by rates of excretion, for these rates varied in different animals. In our experiments the comparison of the excretion of either dye by the two parts of the kidney, glomerulus and tubule, was done in consecutive periods of a single experiment in the same animal and the contrasting results expressed as simple rates, i.e., mgs. per hour.

In regard to the effect of narcosis of the tubules on the excretion of phenol red, Scheminzky states that in five of twelve experiments although the anesthetic produced its typical effect on all the other urinary constituents, it did not depress the rate of excretion of the dye, as is required to support his hypothesis of tubular secretion. Doses of from two to five times the size which produced these results were required to regularly produce a depression of dye elimination. This is ex-

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in amount equal to any fraction of the total venous flow, the arterial fluid being shunted around the glomerular tuft by way of the arteriae rectae to join the intertubular capillaries. Such anastomoses have been shown by Nussbaum and Hayman to mention only the first and last of a long series of investigators. Under these conditions, which are as proper an assumption as that of Scheminzky, it would be a simple matter to account for the total amount of dye found in the urine as a result of venous rather than arterial perfusion of the glomerular tuft.

[3] As a matter of fact the pressures used by us are even less than the maxima that have been observed existing in the living frog's circulation by many other observers. Hayman gives a figure of 60 centimeters of H_2O as a possible normal aortic pressure, the average being 37 centimeters. Our venous pressure could doubtless be reduced, and this would lessen the danger of a contaminating venous perfusion of the glomerulus, but we have selected the pressure given above as proper to insure a complete circulation around the tubules of the entire kidney.

plained by the assumption that the secretory function of the tubule is more re-
sistant to narcosis than the absorptive process.
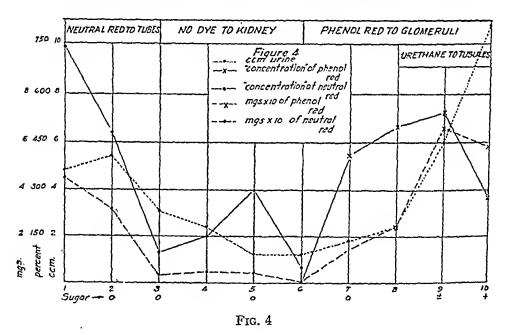
Another explanation is, however, possible and that is that the larger doses of
anesthetic affect more of the renal apparatus than the tubule. In a series of
experiments on experimental nephritis in the frog to be published later, in which
attempts were made to cause selective damage to various parts of the kidney,
great difficulty was encountered in damaging any one part of the renal unit without
affecting to some degree the other elements. The glomeruli, the tubules, and
especially the vascular system are all susceptible to damage when strong toxic
agents are administered even with great care to any one portion of the kidney, and
under these conditions of wide spread damage its function is altered in such a
complex way that it is impossible to judge as to the part played by any single
structure in the production of the total effect.

Our experiments on the winter frogs have also shown an accessory
mechanism for the elimination of phenol red by the kidney. As the
following table shows, although the increases after tubular damage in
rates of water and dye excretion are in most cases of similar magni-
tude, occasionally the dye elimination is increased to a far greater
degree.

Increase in water elimination	Increase in dye excretion
2.5	2.7
8.2	7.3
2.3	2.2
2.5	3.2
5.	22.0

The last figure of this group, which is from an experiment in which
there was a long period of dye absorption before the administration
of the anesthetic to the tubules, it would appear that there is some
other source of dye than that responsible for water output. As our
experiment 3 shows, this added source is doubtless the dye which has
been absorbed from the lumen and stored in the tubule cells. An
"anomalous secretion" of such stored dye is apparently possible
under unusual conditions, such as, for example, when the concen-
tration of dye in the lumen of the tubule falls far below that in the
tubule cells, or when these cells are damaged and no longer able to
hold it in concentrated form. Here again the phenomenon is best
observed in winter frogs for their tubule cells are more highly charged
with dye as a result of their excessive absorptive activity.

Our experiments on the activity of the winter frog's kidney also add a further demonstration of the difference in the manner in which the kidney handles neutral red and phenol red. In such animals with neutral red the concentration factor is usually low when the rate of excretion has fallen to a low figure. With phenol red, the concentration factor is always greater than 100 percent, and often considerably larger, even though the rate of excretion is small. This difference also may be explained by our previous theory of tubular and glomerular excretion of the two dyes. The method of concentration of neutral red is a single mechanism in that it is a function of the tubule alone,

FIG. 4

namely tubular excretion of dye and tubular absorption of water. In the case of phenol red the concentration of the dye is the result of the activity of at least two mechanisms, glomerular excretion and tubular absorption of water and to a lesser degree of dye. An increased activity of the latter may therefore reduce the amount of dye excreted into the final urine without decreasing its concentration, since it is reasonable to suppose that water is absorbed more readily than the dye. The method of "anomalous secretion" of phenol red which we have just described may also aid in producing the result of a high concentration but under these conditions the rate of excretion is apt also to be high.

CONCLUSIONS

1. A method of conservation has been demonstrated in the kidneys of winter frogs.

2. The mechanism of this conservation is an increase in the absorptive function and a decrease in the excretory activity of the tubular epithelium.

3. The excessive absorptive process may be depressed by various means. Such a depression in the tubular activity is followed by large increases in the rate of excretion of water, salts and, if present in the urine, phenol red.

4. Further evidence is thus obtained which supports the theory that phenol red is excreted chiefly through the glomeruli.

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BACTERIOLOGY OF THE BLOOD AND JOINTS IN RHEUMATIC FEVER*

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Although thirty years have elapsed since a streptococcus was first reported in the lesions of rheumatic fever, the etiology of this disease still remains in doubt. It is true, of course, that the streptococcus is now looked upon by many as the exciting agent, but final acceptance of the streptococcal theory is far from being universal.

When one seeks for an explanation of this disagreement, the answer is not hard to find. From time to time streptococci have been found both in the blood and in the joints of patients with acute inflammatory rheumatism, but rarely with sufficient frequency to justify the conclusion that they were the cause of the disease. Indeed, several criticisms can fairly be made of previous investigations in this field; the number of cases studied has usually been small; some authors have failed to state the exact percentage of positive cultures obtained; and control cultures have not been made on individuals free from rheumatic fever.

Triboulet and Coyon (1), in 1897, cultivated a diplococcus from five living cases of rheumatic fever, and at autopsy on one fatal case. Later (1898) they (2) described the production of mitral endocarditis in rabbits by the intravenous injection of a diplococcus isolated from the blood of a patient with rheumatic fever. In another article Triboulet and Coyon (3) reported that in 11 consecutive cases of rheumatic fever they had isolated a diplococcus from the bloodstream.

Apert (4) in 1898, using the methods of Triboulet and Coyon, took blood cultures on two cases of chorea. One yielded a diplococcus similar to the strains recovered by these investigators. In 1899, Westphal, Wassermann and Malkoff

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(5) made a bacteriologic study of a fatal case of rheumatic fever with chorea and endocarditis. A streptococcus was recovered from the blood, the brain and the heart valves. Experiments were conducted on 80 rabbits with the production of arthritis in a considerable number.

In 1900, Poynton and Paine (6) demonstrated diplococci in eight cases of acute rheumatic fever. Five of the eight cases, however, were fatal, and in these five the cultures were obtained at the post mortem table. In three of the eight cases blood cultures were taken; two out of the three were positive for diplococci. In the remaining five cases, diplococci were isolated from pericardial fluid, vegetations on the heart valve or from the tonsils. When injected into rabbits these diplococci produced arthritis, valvulitis and pericarditis.

Philipp (7), in 1903, took blood cultures on 31 cases of rheumatic fever and obtained entirely negative results. He concluded that rheumatic fever is a specific infectious disease of unknown etiology.

Loeb (8), in 1908, took blood cultures on 45 cases of rheumatic fever. In eight patients a streptococcus was obtained which morphologically and culturally corresponded to that described by Poynton and Paine (6) and other workers.

In 1908 Beattie (9) examined three cases of fatal rheumatic fever at the post mortem table. In all of them cultures from the heart's blood remained sterile. In all three cases, however, streptococci were grown from pieces of synovial membrane. These strains were identical in their cultural characteristics, and differed in no way from Poynton and Paine's "streptococcus rheumaticus."

In 1913 Rosenow (10) reported the isolation of streptococci from the joints in seven out of eight cases of acute rheumatic fever. The same author (11), in 1914, recovered streptococci from the bloodstream in four out of seven patients with rheumatic fever. Rosenow divided his streptococci into three groups, according to their effect on blood agar: 1. Those producing green; 2. Those producing slight hemolysis; 3. Those producing no perceptible change. Rosenow claimed that under certain conditions any of the three varieties could be converted one into the other. When these streptococci were injected into rabbits, they induced arthritis, endocarditis and pericarditis.

Herry (12), in 1914, undertook an elaborate study of rheumatic fever based on sixty cases. Forty-three out of the sixty cases yielded positive blood cultures, in every case a diplococcus similar to that described by Poynton and Paine. Four out of five joint cultures were positive for streptococci, and seven pleural fluids all yielded streptococci. Altogether 47 of the 60 cases (78.3 per cent) yielded a streptococcus from some one of these sources. Herry found that arthritis, myocarditis and endocarditis were readily produced in rabbits by intravenous injections of these streptococci.

Swift and Kinsella (13), in 1917, cultured the blood from 58 cases of rheumatic fever and obtained streptococci from seven, or 12 per cent, of them. The joints were cultured in 25 cases with entirely negative results. All of Swift and Kinsella's streptococci produced green on blood agar plates, but no strict *biological*

relationship could be established between these strains by complement-fixation tests. No agglutination tests were made.

In 1925, Clawson (14) isolated streptococci from the blood in a "relatively high percentage of cases of rheumatic fever." His technic for blood cultures differed from that of his predecessors in several respects: 1. Fifty c.c. of blood were collected for culture. 2. Only the clot was used for cultivation. 3. Cultures were observed for one month before being discarded. Altogether twenty strains of streptococci were studied. With one exception they all produced methemoglobin after a period of cultivation. The exceptional strain was a typical streptococcus hemolyticus. By injecting these streptococci into rabbits, Clawson found that lesions similar to those occurring in human rheumatic fever could be produced experimentally.

Zinsser and Yu (15) (1928) reported the results of cultures on two cases of rheumatic fever and two cases of rheumatic carditis. In one rheumatic fever patient, intra-vitam blood cultures revealed both an alpha and a gamma strain of streptococcus. The other rheumatic patient died and at autopsy an alpha streptococcus was recovered from the myocardium and from the pericardial fluid. Both of the patients with carditis died. Cultures from the blood of both these patients were sterile, but post mortem cultures from the spleen in each instance yielded a streptococcus.

Surányi and Forró (16) (1928) took blood cultures on 25 cases of "polyarthritis" and obtained green streptococci in 17, or 68 per cent, of the series. The blood was first hemolyzed in citrate solution and the sediment cultured in broth tubes. In contrast to these positive findings is the recent study of Nye and Seegal (17) (1929) on the bacteriology of the blood in rheumatic fever. Fifty cubic centimeters or more of blood was taken in nearly every instance. Twenty-five cases were cultured in this way with entirely negative results.

Small (18) and Birkhaug (19), whose recent studies (1927) on the etiology of rheumatic fever have attracted considerable attention, have not been particularly interested in the bacteriology of the blood and joints in rheumatic fever, but have investigated the gamma type of streptococcus in relation to its presence in the throats of patients with rheumatic fever. Small's original R1 "streptococcus cardioarthritidis" was isolated from the blood, but most of his subsequent strains were obtained from the throats of rheumatic fever patients. Of Birkhaug's 27 strains of "non-methemoglobin-forming streptococci," only three were isolated from the blood, the remainder from tonsils, feces, etc.

It is evident that investigators have met with varying degrees of success in their attempts to recover streptococci from the blood and joints in rheumatic fever. It is significant, however, that those who have recovered streptococci from rheumatic fever patients have almost invariably recovered the same type of streptococcus, namely the streptococcus viridans. Furthermore, although these streptococci

have presented very much the same appearance morphologically and culturally, the trend of evidence is against their comprising one biological group. In addition to the streptococcus viridans, an indifferent streptococcus similar to that described by Small and by Birkhaug has occasionally been isolated.

The Present Study

The present investigation, initiated in the Spring of 1928, followed as a natural corollary to our study on the bacteriology of the blood and joints in chronic infectious arthritis, the results of which have been recently published (20). If streptococci could be isolated frequently from the blood and from the joints in infectious arthritis, it seemed reasonable to suppose that they could also be found in the blood and joints in rheumatic fever, a disease which, though differing from infectious arthritis in many respects, possesses with it certain points of similarity.

It was decided that only those cases of rheumatic fever that showed fever and definite joint manifestations would be studied. Altogether, sixty such patients have been included in this investigation. In almost every instance cultures were taken during the febrile stage and usually before salicylates had been given. During the Spring of 1928, 29 cases were studied; during the Spring of 1929, 31 cases. Most of the patients in the series were adults,* but through the kindness of Dr. Charles Hendee Smith we also had access to the children's wards at Bellevue Hospital. Unfortunately, many of the rheumatic children showed no active joint manifestations, and could therefore not be included in the series.

Methods

The technical methods employed are submitted in detail:

(1) *Broth for Blood Cultures.*—Fresh beef heart free from fat is passed through a meat grinder and weighed. For every 1000 grams of meat one liter of tap water is added. The mixture is allowed to stand in the ice-box over night. In the morning, it is heated to 20 deg. C., and filtered through a flannel bag. It is

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* We are very much indebted to Dr. Van Horne Norrie, Dr. John Wyckoff and Dr. Charles Hendee Smith for the privilege of using material from their services in Bellevue Hospital.

then boiled over a low flame for one hour with frequent stirring. After filtering through filter paper, water is added in sufficient quantity to make up for loss by evaporation. One per cent bacto-peptone (Difco standardized) and 0.5 per cent sodium chloride are added. The broth is next placed in the Arnold sterilizer at 100 deg. C. for ten minutes to dissolve the salt and peptone. The pH. is adjusted to 7.8 with 2N sodium hydroxide (Kahlbaum) using a La Motte block comparator with phenol red as indicator. The solution is placed in the Arnold at 100 deg. C. for one hour and again filtered through filter paper. The pH. is again taken while the broth is still hot, and is usually found to have fallen to 7.6. If it should fall below 7.6 a further adjustment of the pH. is necessary followed by another hour in the Arnold. The final pH. of the broth should be 7.6. It is poured into 100 c.c. bottles (50 c.c. to each bottle) and sterilized in the Arnold for 30 minutes at 100 Deg. C. on three successive days. The broth is then incubated for 24 hours to test for sterility.

(2) *Blood Broth for Joint Cultures.*—Broth for joint cultures is prepared in the same way. The broth is then poured into ordinary culture tubes (8 c.c. to each tube) and sterilized in the Arnold for 30 minutes at 100 deg. C. for three successive days. To each tube of broth is now added 0.1 c.c. defibrinated rabbit's blood. The broth is then incubated for 24 hours to test for sterility.

(3) *Preparation of Agar.*—Beef heart free from fat is passed through the meat grinder and weighed. For every 1000 grams of meat two litres of water are added. The mixture is allowed to stand in the ice-box over night. In the morning it is heated to 20 deg. C. and filtered through a flannel bag. It is then boiled over a low flame for one hour with frequent stirring. After filtering through filter paper, water is added in sufficient quantity to make up for loss by evaporation. One per cent bacto-peptone (Difco standardized), 0.5 per cent sodium chloride, and 1.5 per cent bacto-agar (Difco standardized) are added. The mixture is placed in the Arnold at 100 deg. C. for 30 minutes to dissolve the salt, peptone and agar. The pH. is adjusted to 7.8 with 2N sodium hydroxide (Kahlbaum). The solution is placed in the Arnold at 100 deg. C. for one hour. It is then passed through a Sharples centrifuge to remove the sediment. The pH. is again taken while the agar is still hot, and it is usually found to have fallen to 7.6. If it should fall below 7.6, a further adjustment of the pH. is necessary, followed by another hour in the Arnold. The final pH. of the agar should be 7.6. It is poured into test tubes (8 c.c. to each tube), and sterilized in the Arnold for 30 minutes at 100 deg. C. on three successive days, allowing the media between sterilizations to stand at room temperature. Tests for sterility are made by incubation for 24 hours.

(4) *Technic for Blood Cultures.*—The syringes and needles used for taking the cultures are enclosed in glass tubes and sterilized in the dry sterilizer for two hours at 150 deg. C. The skin is prepared with two coats of iodin and one of alcohol. Twenty c.c. or more of blood are taken from a vein in the arm. The blood is divided between two culture tubes, allowed to clot, and put in the ice-box over

TABLE 1

Résumé of 60 Cases of Acute Rheumatic Fever Cases Studied in 1928

Patient	Age	Sex	Previous Attacks	Preceding Sore Throat	No. of Days Ill Before Adm.	Joints* Involved	Cardiac Involvement §	Max. Tem.	W.B.C.	Blood Culture† 1st	Blood Culture† 2nd	Remarks
B-929	22	M	1		21	W. K. A. H. S.	Systol. Murmur	104.2		—		
B-932	20	M	2	Yes	14	S. A. K.	Endocard.	100.4		—		
B-856	48	M	Many		21	A. K. F.	Auricul. Fibrill.; Myocard.	103.2	14,200	RB-7		
B-375	9	F		Yes	10	A. K. T.	Endocard.	104.2	11,800	RB-2		Pyelitis.
B-377	32	M	2		21	A. K.		101.0	10,800	RB-37		
B-373	7	M			3	K. A.	Endocard. (later)	103.3	11,000	—		
B-356	9	F		Yes	30	F. K. S. W.	Endocard.	104.0	23,400	RB-6	—	Rheumatic nodules over olecranon.
B-372	11	M	1		6	A. T.	Systol. Murmur	100.6	10,100	RB-3		
B-924	28	F	2		10	K. S. W. F. A.		104.2	16,600	RB-1	—	Acute nephritis.
B-434	28	F			6	K. A. W. H. T.		102.8	12,850	—		
B-911	18	F		Yes	7	E. W. S. K.		103.4	13,900	—		
B-900	31	F			48	F. T. S. K. A.	Pericard.	105.4	26,600	RB-38		Fever and signs of pericarditis until death 18 days after admission; no sign of endocarditis.
B-923	20	M	1		14	S. A.	Endocard.	101.6		—		
B-949	37	M			14	K. E.		102.0	16,500	—		
B-2161	29	M	1	Yes	3	K. A. S.	Endocard.	103.0	11,400	—		

Case	Age	Sex			No.	Initials	Diagnosis	Temp.	W.B.C.	Culture	RB-4 (PM)	Remarks
B-948	24	M	1		9	H.	Peri. Endo. & Myo. card.	104.0	11,450	—		Died; anatomic diagnosis. (1) Serafibrinous pericarditis. (2) Rheumatic endocarditis. (3) Rheumatic nodules.
B-903	23	F		Yes	5	F. K. F. S.	Systol. Murmur	103.6	15,400	—		
B-909	19	M		Yes	9	K. W. E.	Systol. Murmur	104.0	12,000	—		
B-917	21	M			14	K. W. A. E.	Gallop rhythm	101.8	23,000	—		Acute nephritis; p-r. interval 0.2; left hospital with pulse rate 110, temperature 99.6.
B-902	25	M			5	K. A. S. E.		102.2	16,000	—		
B-921	35	F	1		6	A. K. W. F.	Myo. Endo. & Pericard.	104.6	10,400	—		
B-350	9	M	3		6	K. H. E.	Endocard.	102.8	13,600	—		Furunculosis.
B-918	44	F	1		8	H. K. A. W.		103.2	15,600	—		Acute otitis media.
B-919	46	F	1	Yes	22	S. A.	Endocard. Myo-card.	104.0	13,200	—		
B-348	11	M			42	K.	Endocard.	101.0	8,000	—		Schönlein's disease; many large patches of purpura on legs.
B-40	35	F			10.	K. A.		105.4	15,500	RB-5	—	
B-1423	20	F			14	W. A. K. F.		103.0		—		
B-1412	29	M			4	A. K. W.		103.4		—		
B-1433	29	M			3	K. S. F. A.		102.0		—		

Cases Studied in 1929

Case	Age	Sex			No.	Initials	Diagnosis	Temp.	W.B.C.	Culture	RB-4 (PM)	Remarks
H. E.	22	F	4		5	F. K. W.	Endo. Peri. & Myocard.	105.6	20,000	RB-10	—	No petechiae; beginning decompensation; spleen not enlarged; p-r. interval 0.24 right axis deviation; inverted T 1st & 2nd.
B-963	23	M		Yes	5	K. A. W.	Endocard.	105.0	15,800	RB-23		

TABLE 1—Concluded

Patient	Age	Sex	Previous Attacks	Preceding Sore Throat	No. of Days Ill Before Adm.	Joints* Involved	Cardiac Involvement §	Max. Tem.	W.B.C.	Blood Culture‡ 1st	Blood Culture‡ 2nd	Remarks
B-964	25	F			14	K. A. S.		101.0		RB-22		
B-402	46	M		Yes	5	K. A. S. H.		101.4		RB-28		
B-384	29	F		Yes	42	F. E. K. S.	Endocard.	101.8		RB-14		
B-861	38	M	1		13	W. K. A.		104.6	16,900	RB-19		
B-965	48	M	Many		11	K. A. W. E.	Pulsus Bigem; Myocard.	102.6		RB-20		
B-961	17	M	Many	Yes	30	S. E. K.	Endo. & Pericard.; Auricul. Fibrill.	104.0	18,600	RB-24		Died suddenly; no autopsy.
B-1495	20	M	2	Coryza	14	K. E. A.	Endocard.	102.4		RB-35		Chorea as a child.
B-966	21	F	1	Yes	21	K. A. W.	Endocard.	102.0		—		
N. F.	30	F		Yes	30	K. E. A.	Endocard.	102.4		RB-33		
P. S.	35	F	1	Yes	30	K. S. W. F. E.	Endocard.	104.0		RB-34	{	S. V. (RJ-34) recovered from joint culture.
R. G.	18	M	1	Yes	4	A. K. V.	Peri. Endo. & Myo-card.	104.8		RB-32		Pleuritis with effusion p-r. interval 0.22.
D. S.	44	M	1	Yes	5	K. W. E. S.	Endo. & Pericard.; Auricul. Fibrill.	103.0		RB-25		S. V. (RJ-25) recovered from joint culture.
B-1497	43	F			2	S. E. W. F. K.	Systol. Murmur	102.8		RB-21		
S. T.	23	M			5	W. E. H. K. A.		101.8		RB-11		
R. C.	65	M	2		7	K. E. W. A.	Myocard. Endo-card.	103.2		—		S. V. (RJ-36) recovered from joint culture.
B-400	38	M		Yes	5	K. S. E. A.		103.8		RB-9$_1$	RB-9$_2$	S. V. (RJ-17) recovered from joint culture.
A. K.	29	M		Yes	6	K. A.		100.6		RB-17		

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	Age	Sex			Days	Joints	Cardiac	Temp.	W.B.C.	Strain		Remarks
M. B.	46	F	1		7	K. S. E. A.	Endocard.	103.4		RB-29		p-r interval 0.15.
R. Z.	32	F	1	Coryza Yes	60	W. K. F.	Myocard.	100.8	8,800	RB-31	—	Epilepsy.
N. M.	14	M			5	A. W. K.	Endocard.	102.4	15,000	RB-13		
S. F.	9	F	1	Yes	3	A. K.	Endocard.	103.0	9,100	RB-27		S. V. (RJ-16) recovered from joint culture.
L. M.	47	M			3	F. A. W. K.	Systol. Murmur	104.0	6,900	RB-16	—	
A. F.		M	1	Yes	1	S. W. K.	Endocard. Myocard.	103.8		RB-18		
B-2304	28	F	Many	Yes	30	W. K.	Endocard.	103.4		RB-15		
A. C.	13	F	1	Yes	7	K. A.	Endocard.	100.8		—		
B-953	49	M	Many		7	A. K. S. H.		102.8	16,600	RB-8		Wassermann ++++. Joint culture negative.
B-1506	30	M	2		30	K. A. W. S.		101.8		RB-26		Joint culture negative.
C. W.	19	M	2		2	K. S. H.	Endocard.	101.9		—		
J. M.	27	M			3	K. S. E. F.		103.6		—		

* A, indicates ankle; E, elbow; F, fingers; H, hips; K, knee; S, shoulder; T, toes; W, wrist; V, vertebrae.
† Cultures positive for streptococci are indicated by the strain numbers. — = no growth.
§ Systol. murmur = Systolic murmur; Endocard. = Endocarditis; Pericard. = Pericarditis, Myocard. = Myocarditis; Auricul. Fibrill. = Auricular Fibrillation.

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night. On the following morning, the clots are loosened with a glass rod and the tubes placed in the centrifuge. After centrifugalization, the serum is carefully removed with a pipette. The clots are broken up with glass tubes one-quarter inch in diameter, drawn up in the tubes and transferred to two bottles, each containing 50 c.c. of broth. The bottles are then covered with paper caps and placed in the incubator at 37 deg. C. for one month. During the month, the bottles are opened at five day intervals, and sub-cultures made in both blood-agar pour plates and blood broth tubes, using one-tenth c.c. of the original broth culture in each case. If no growth has been found at the end of a month, the original broth bottles are centrifugalized and the sediment cultured in fresh blood broth. Sub-cultures are made from the latter on blood agar plates several

TABLE 2

Summary of Bacteriological Findings in Blood and Joint Cultures of Patients with Acute Rheumatic Fever (1928 and 1929 Series)

Organism found	Rheumatic fever		Controls	
	Blood	Joints	Blood	Joints
Strep. viridans............................	33	5	1	0
Strep. hemolyticus........................	1	0	0	0
Strep. anhemolyticus......................	1	0	0	0
Total showing strep.......................	35	5	1	0
Sterile....................................	25	2	65	13

days later, and if no organisms are found, the bottles are discarded and the culture considered negative. All transfers and manipulations are carried out under a hood.

(5) *Technic for Joint Cultures.*—Syringes and needles for aspiration are prepared in the same way as for blood cultures. The knee joint has been used for obtaining joint fluid. The skin over the joint is prepared with two coats of tincture of iodin and one of alcohol. Two per cent novocain is injected down to the capsule to render the parts anesthetic. A 20-gauge needle and a 20 c.c. syringe are usually employed for the aspiration. The fluid from the joints is cultured in three or four blood broth tubes, using varying amounts for each tube. The tubes are placed in the incubator for one month. Sub-cultures are made every few days in blood-agar pour plates and in blood-agar streak plates. Streptococci usually make their appearance during the first week, but the cultures are not pronounced sterile until they have been in the incubator one month.

Results of Blood Cultures

Protocols of the 60 patients on whom blood cultures and, in some instances, joint cultures were made, are listed in Table 1. The age of

these patients varied from 7 to 65 years.   Thirty-four gave a history of one or more previous attacks of rheumatic fever.   In 26 patients a history was given of a preceding sore throat or coryza.   Every patient in the series gave a history of joint manifestations, and in nearly every case several joints were still obviously inflamed at the time the cultures were taken.   Every case showed fever varying from a maximum of 100.4 to 105.6.   Leucocytosis was present in most of the cases on whom counts were made.   Only three patients in the

TABLE 3

Patients with Rheumatic Fever Subjected to Two Blood Cultures

Patient	1st Blood Culture	2nd Blood Culture	No. of Days Between Cultures	Status of Disease at Time of 2nd Blood Culture
B-356	+	—	5	Active
B-924	+	—	10	Inactive
B-40	+	—	2	Subsiding
B-948	—	+ (P.M.)	2	Patient dead
H. E.	+	—	57	Inactive
P. S.	+	—	21	Subsiding
R. Z.	+	—	50	Inactive
L. M.	+	—	21	Subsiding
B-400	+	+	9	Active

Active indicates that the patient had high fever and joint lesions.
Subsiding indicates that the fever was subsiding and the patient recovering from the attack.
Inactive indicates that the patient had no fever and was out of bed.

series of 60 cases died.   One came to autopsy and showed no evidence of bacterial endocarditis.   In the other two fatal cases, the opinion of all those who studied them clinically was that death was due to a serofibrinous pericarditis and not to a bacterial endocarditis.
Of the 29 cases studied by blood culture in 1928, nine, or 31 per cent, were found, after varying periods of incubation, to contain a gram-positive micrococcus in pure culture.   Of the 31 cases studied in 1929, 26 or 83.9 per cent yielded a micrococcus in pure culture. The organisms appeared in pairs or short chains and had all the morphologic characteristics of streptococci (Table 2).   The higher

percentage of positive cultures obtained in 1929 is probably traceable in large part to improvements in technic, though a personal equation was also present, the cultures in 1928 having been examined by one of the writers and the cultures in 1929 by another.

Nine patients studied in this series were subjected to two blood cultures (Table 3). Of these patients 8 showed streptococci in the

TABLE 4

Classification of Patients Studied by Blood Cultures

	No. of cases Studied by Blood cultures	Total	Streptococcus in Blood cultures
Acute Rheumatic Fever.....................	60	60	35
Controls:			
Fever of unknown origin (Blood cult. positive).............................	1		
Degenerative arthritis.....................	23		
Gonococcal arthritis......................	4		
Chronic myositis.........................	7		
Chronic neuritis.........................	3		
Gout....................................	2		
Chorea..................................	2		
Tuberculous adenitis.....................	2		
Diabetic neuritis........................	1	66	1
Convalescent pneumonia.................	7		
Iridocyclitis.............................	1		
Weak foot..............................	1		
Chronic colitis..........................	1		
Sciatica................................	1		
Gastric neurosis.........................	1		
Chronic nephritis........................	1		
Neurasthenia............................	3		
Normal individuals......................	5		

first culture; two yielded streptococci in the second culture. All blood cultures taken when the disease was subsiding or inactive were sterile.

Other Bacteria Isolated from Blood Cultures.—Some type of streptococcus was the only organism consistently recovered from blood cultures. In one instance blood cultures yielded a diphtheroid bacillus, but in view of the frequency in which this organism is found as a

contaminant, no significance was attached to its presence. All grossly contaminated cultures were discarded.

Control Blood Cultures.—It seemed important to check these observations on the blood of patients with acute rheumatic fever by a series of blood cultures on patients suffering from other diseases and on normal individuals. Altogether, 66 controls were studied by means of blood cultures, the technic being similar in all respects to that used in acute rheumatic fever (Table 4).

All the controls, with one exception, yielded sterile blood cultures.

The exceptional case (B-284) was that of a man, aged 53, who six hours before admission began to have muscular pains particularly in the back and legs asso-

TABLE 5

Classification of Patients Studied by Joint Cultures

	No. of cases Studied by Blood culture	Total	Streptococcus in Joint cultures
Acute Rheumatic Fever....................	7	7	5
Controls:			
Degenerative arthritis....................	4		
Gout...............................	1		
Chronic synovitis........................	3	13	0
Traumatic synovitis.....................	4		
Osteitis fibrosa cystica..................	1		

ciated with malaise and high fever. There were no other symptoms. The past history was negative. Physical examination showed a flushed feverish man with a reddened pharynx. The remainder of the examination was negative. X-rays of the sinuses, lungs and gall-bladder revealed no evidence of disease. W.B.C. 6200; Widal negative; urine negative. The temperature on admission was 104.6. It came down to normal on the fifth day. Then it gradually rose again, and ran an irregular course between 100 and 102 for another four weeks, when it came down to normal and remained there. The final diagnosis on this patient was "Fever of unknown origin." A blood culture taken on the day of admission yielded a pure culture of streptococcus viridans.

Results of Joint Cultures

In seven cases of acute rheumatic fever, the opportunity presented itself for culturing the synovial fluid from one of the affected joints.

In every case, the knee was the joint selected for puncture, and in every instance 5 to 20 c.c. of slightly cloudy, straw-colored fluid was removed. Of the seven cases, five yielded a streptococcus in pure culture after varying periods of incubation (Table 5). Neither of the two patients on whom sterile joint cultures were obtained were presenting very active symptoms at the time the punctures were made.

Control Cultures.—These results were checked with joint cultures of patients suffering from other diseases (Table 5). Altogether, 13 controls were studied by means of joint cultures, the technic being similar in all respects to that used on patients with acute rheumatic fever. All the controls yielded sterile cultures.

Cultural and Biologic Characteristics of the Streptococci Isolated from Blood and Joint Cultures

The average time of appearance of streptococci in the blood cultures was approximately 17 days; in joint cultures, 8 days. In one instance the blood culture had been in the incubator six weeks before streptococci could be demonstrated. On the other hand there were exceptional cases in which the streptococci appeared in three or four days. In many instances, the first colonies observed on the blood agar sub-culture plates failed to grow when seeded into blood broth tubes. It was often necessary to return to the original broth cultures several times before successful transfer of colonies to blood broth could be achieved.

The streptococci first make their appearance in broth as pairs or very short chains, the chains becoming longer as the organisms became more accustomed to laboratory mediums. Generally, the organisms grow very poorly for the first few generations, necessitating frequent transfers of cultures. In blood agar sub-cultures, both superficial and deep colonies are usually recognizable after 24 to 48 hours of incubation.

The streptococci encountered in the blood and joint cultures with two exceptions belong to the alpha streptococcus or streptococcus viridans group. One exception proved to be a typical hemolytic or beta streptococcus, while the other was classified as a non-hemolytic or gamma streptococcus. All the streptococci isolated were gram-positive and insoluble in bile.

Morphologically, the streptococcus viridans strains usually appear as very small, round or slightly oval cocci, which in liquid mediums form pairs or very short chains. On blood agar plates, after 48 hours incubation, deep colonies appear as minute, biconvex grayish colonies surrounded by a very small zone of incomplete hemolysis that contains specks of green pigment. The amount of methemoglobin formation varies considerably with different strains. A few strains show a large amount of green pigment, but as a general rule, the amount of green produced is small.

No hemolysis of the blood occurs in blood broth cultures after 48 hours incubation. The broth becomes turbid and takes on a slightly greenish tinge.

The strain of streptococcus hemolyticus cultivated from one patient grows readily on all the usual laboratory mediums. In blood agar, after 48 hours incubation, deep colonies appear as moderate-sized, biconvex grayish colonies, surrounded by large zones of complete hemolysis. The indifferent streptococcus isolated from one patient appears as a small, round or slightly oval coccus, which in liquid mediums form very short chains. On blood agar, after 48 hours of incubation, deep colonies appear as small, biconvex grayish colonies with no change in the surrounding medium. There is no hemolysis of the blood in blood broth cultures.

Sugar Fermentations.—Sugar fermentations were carried out on 32 strains of streptococci isolated from the blood, and five strains isolated from the joints (Table 6).

The tests were made in 5 c.c. of sugar free broth, to which 0.1 c.c. of a ten per cent solution of one of the sugars had been added. The pH. of the sugar mediums was uniformly 7.6. Each tube was heavily inoculated with an actively growing 24-hour broth culture. The tubes were allowed to incubate for five days, and the amount of acid formation determined by the pH. method. Tubes that gave only a small amount of fermentation were allowed to incubate for an additional 48 hours, but no further amount of acid formation was noted in any case.

Thirty-four of the 37 strains fermented lactose, mannit and salicin. According to Holman's classification, this group would be classified as streptococcus fecalis. It included all but one of the streptococcus viridans strains. Three of the 37 strains (RB-2, RB-5 and RB-6) fermented salicin and lactose, but had no effect on mannit. Of these three strains, two were obviously members of a different biological group. Strain RB-2 was a streptococcus hemolyticus and RB-5 was

a gamma or indifferent streptococcus. Only two strains in the entire series produced acid in inulin, and even in these two the change in the pH. was so slight (7.2) that the strains could hardly be called inulin fermenters.

It is significant that three strains of streptococcus viridans isolated from joints corresponded in their fermentation reactions with the strains isolated from the blood in the same patients. Strains RB-9₁

TABLE 6

Sugar Reactions of Streptococci

Strain	Dex-trose	Salicin	Lac-tose	Man-nit	Inulin	Strain	Dex-trose	Salicin	Lac-tose	Man-nit	Inulin
RB-1	6.2	6.8	6.2	6.4	7.6	RB-22	6.0	6.0	6.0	6.0	7.6
RB-3	6.2	6.8	6.0	6.0	7.6	RB-23	6.0	6.0	6.0	6.0	7.6
RB-7	6.5	6.8	6.0	6.2	7.6	RB-24	6.2	6.8	6.0	6.2	7.6
RB-8	6.0	6.0	6.0	7.0	7.6	RB-25	6.0	6.0	6.0	6.0	7.6
RB-9₁	6.0	6.4	6.0	6.0	7.6	RJ-25	6.0	6.0	6.0	6.0	7.6
RB-9₂	6.2	6.8	6.0	6.2	7.6	RB-27	6.0	6.0	6.0	6.0	7.6
RB-10	6.0	6.0	6.0	6.0	7.6	RB-28	6.0	6.4	6.0	6.0	7.6
RB-11	6.0	6.0	6.0	6.0	7.6	RB-29	6.0	6.0	6.0	6.0	7.6
RB-12	6.0	6.0	6.0	6.0	7.6	RB-31	6.0	7.0	6.0	6.0	7.6
RB-13	6.0	6.0	6.0	6.0	7.6	RB-32	6.0	6.0	6.0	6.0	7.6
RB-14	6.0	6.4	6.4	6.2	7.6	RB-34	6.0	6.0	6.0	6.0	7.6
RB-15	6.0	6.0	6.0	6.0	7.6	RJ-34	6.0	6.0	7.0	6.0	7.6
RB-16	6.0	6.0	6.0	6.0	7.6	RJ-36	6.0	6.6	6.0	6.0	7.6
RJ-16	6.0	7.0	6.0	7.0	7.6	RB-26	6.0	6.0	7.0	6.0	7.2
RJ-17	6.0	6.0	6.0	6 0	7.6	RB-35	6.0	6.0	6.0	6.0	7.2
RB-18	6.0	6.6	6.0	6.0	7.6	RB-2	6.0	6.0	6.0	7.6	7.6
RB-19	6.2	6.4	6.0	6.2	7.6	RB-5	6.0	6.8	6.2	7.5	7.6
RB-20	6.0	6.0	6.0	6.0	7.6	RB-6	6.0	6.0	7.0	7.6	7.6
RB-21	6.0	6.0	6.0	6.0	7.6						

The figures in the table indicate pH reading after 5 days incubation.
Readings of 7.6 = no acid formation.
Readings of 7.0 or below = definite acid formation.

and RB-9₂, isolated at different times from the blood of Case B-400, gave identical sugar reactions.

Agglutination Reactions.—In order to determine whether there was any biologic relationship between the streptococci which had been isolated from the blood and joints of patients with acute rheumatic fever, cross agglutination tests were made with 16 serums from

rabbits immunized against 16 strains of streptococci.   Fifteen of these strains were from the blood or joints of patients with rheumatic fever.   The sixteenth strain was a streptococcus (AB-4) isolated from the blood of a case of chronic infectious arthritis.

The 16 immune serums were tested for agglutinins against each of the 35 strains of streptococcus recovered from patients with rheumatic fever and against one strain from a case of chronic infectious arthritis. In addition the serums were tested against a streptococcus viridans recovered from the blood of a patient with subacute bacterial endocarditis.

The agglutination tests were carried out as follows:

A 24-hour broth culture was killed by heating at 56 C. for two hours, and the centrifugalized sediment re-suspended in physiological salt solution.   The rabbits received 5 c.c. daily for five days.   After five days rest, they were given five further injections (10 to 15 c.c. each) at daily intervals.   After another five days rest, tests for the agglutination titer were made.

By this method, the serums when collected were usually found to be rich in agglutinins.   However, in a few instances, it was necessary to give additional injections of streptococci.   All the serums showed a minimum agglutination titer of 1:2560.

The antigens for the agglutinations were prepared by growing the streptococci in plain broth for eighteen hours.   A diffuse growth was obtained in this way with every strain.   0.5 c.c. of the antigen was mixed with 0.5 c.c. of the various dilutions of immune rabbit's serum.   The agglutinations were carried out to a titer of at least 1:2560, and sometimes to a titer of 1:655,000.   As controls, rabbit's serum withdrawn before immunization was tested against the streptococci. Another control consisted of 0.5 c.c. of sterile broth and 0.5 c.c. of the bacterial antigen.   The agglutination tubes were left two hours in the water bath at 56 C. They were then placed in the refrigerator, and readings were made the following morning.

Examination of Table 7 reveals the fact that these green streptococci recovered from the blood and joints of rheumatic fever patients show a definite tendency to fall into biological groups.   We have indicated this grouping in the chart by rectangles.   These streptococcus groups differ from pneumococcus groups in that there is more tendency to partial cross-agglutination between the groups.   This is particularly noticeable in the case of the dominant group in the upper left corner of the chart.   There are several strains listed just below this group

TABLE 7

Agglutination Tests

Agglutination Reactions with Serums of Rabbits Immunized against Fifteen Strepto-
coccus Strains from Rheumatic Fever and One from Chronic Infectious Arthritis

Strains	Serum RB-19	Serum RB-24	Serum RB-9	Serum RB-7	Serum RB-25	Serum RJ-36	Serum RB-5	Serum RB-16	Serum RB-22	Serum RJ-17	Serum RJ-34	Serum RB-26	Serum RB-4	Serum RB-1	Serum RB-2	Serum AB-4
RB-19	4	4	4	4	4	3	3	0	0	0	0	0	0	0	0	0
RB-24	4	4	4	4	4	4	3	0	0	0	0	0	0	0	0	0
RB-9	4	4	4	4	4	4	3	0	0	0	0	0	0	0	0	0
RB-9(2)	4	4	4	4	4	4	3	0	0	0	0	0	0	0	0	0
RB-14	4	4	4	4	4	3	3	0	0	0	0	0	0	0	0	0
RB-18	4	4	4	4	4	3	3	0	0	0	0	0	0	0	0	0
RB-13	4	4	4	4	4	4	3	0	0	0	0	0	0	0	0	0
RB-7	4	4	4	4	4	3	3	0	0	0	0	0	0	0	0	0
RB-25	4	4	4	4	4	4	3	0	0	0	0	0	0	0	0	0
RJ-25	4	4	4	4	4	3	3	0	0	0	0	0	0	0	0	0
RB-20	3	3	3	3	3	3	3	3	4	0	0	1	0	0	0	0
RB-36	3	3	3	2	4	4	2	3	2	4	0	1	2	2	0	0
RB-3	2	2	3	2	2	3	2	3	4	3	1	0	1	1	0	0
RB-8	2	2	2	2	3	2	2	3	2	4	4	1	2	1	0	0
RB-5	2	2	2	2	3	4	4	3	2	3	1	4	1	1	0	0
RB-30	1	1	2	1	0	0	1	0	2	0	0	0	0	0	0	0
RB-31	2	2	2	2	0	0	2	3	0	0	4	0	0	0	0	0
RB-4	2	1	2	2	0	0	3	0	2	0	0	0	4	2	0	0
RB-15	2	2	2	2	0	0	1	4	0	0	0	0	0	0	0	0
RB-16	0	0	0	0	0	1	2	4	1	1	0	2	1	0	0	0
RJ-16	0	0	0	0	0	2	2	3	1	2	0	1	1	0	0	0
RB-32	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
RB-29	1	1	0	0	0	2	1	4	0	1	2	0	0	0	0	0
RB-28	1	1	0	0	0	0	2	3	0	0	4	0	0	0	0	0
RB-34	0	0	0	0	0	1	0	0	2	0	3	0	0	1	0	0
RJ-34	0	0	0	0	0	2	0	0	2	0	4	0	0	0	0	0
RB-35	0	0	0	0	0	2	0	0	0	0	0	0	0	1	0	0
RJ-17	0	0	0	0	0	3	0	0	0	4	0	0	0	0	0	0
RB-26	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0
RB-2	0	0	0	0	0	0	0	0	4	0	0	0	2	0	4	4
RB-22	0	0	0	0	0	0	0	0	4	0	1	0	2	0	2	1
RB-23	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0
RB-10	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0
RB-27	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0
RB-21	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0
RB-1	0	1	0	1	0	0	0	0	0	0	1	0	3	4	0	0
S. B. E.	0	0	0	0	0	1	2	0	0	3	0	0	4	4	0	0
AB-4	0	0	0	0	0	0	0	0	1	0	1	0	0	0	4	4

For the agglutination tests the following dilutions of serums were used, 1:20
1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560. 4 = Complete agglutination
with sedimentation; 3 = agglutination and sedimentation with slight turbidity;
2 = agglutination without sedimentation, 1 = slight agglutination, 0 = no
agglutination.

RB = Rheumatic fever strains; AB = Chronic infectious arthritis strains;
S. B. E. = Subacute bacterial endocarditis. The larger groups are indicated
with rectangles.

that appear to be closely related to it, though they fail to show complete agglutination reactions. The immune serum of hemolytic streptococcus, RB-2, produced little or no agglutination of any of the other rheumatic fever strains, but strongly agglutinated the strain AB-4 from a case of chronic infectious arthritis. Likewise, there was no agglutination between the immune serum of strain AB-4 and the rheumatic fever strains, except the hemolytic streptococcus RB-2. These two strains appeared to be biologically identical. Two of the immune serums gave a complete cross-agglutination and three a partial agglutination with the strain of streptococcus viridans recovered from a case of subacute bacterial endocarditis.

One of the most interesting features brought out in the agglutination studies was the close biologic relationship between the streptococci isolated from the blood and from the joints of the same patient. From three of the patients in this series, streptococci have been recovered from both the blood and the joints, and in every instance the strain recovered from the joint showed a high degree of cross-agglutination with the strain recovered from the blood.

It is also significant that in the case of patient B-400, two blood cultures, taken nine days apart, yielded streptococci that cross-agglutinated.

Absorption Tests.—Absorption tests were carried out on each immune rabbit serum with the strains that showed complete agglutination, and in several instances where there was only partial agglutination. The method was as follows:

The sediment from 300 c.c. of a 24-hour broth culture of a streptococcus was mixed with 0.5 c.c. of a 1:10 dilution of the immune serum. The mixture was shaken and placed in the water bath for two hours at 56 deg. C. While in the water bath, it was agitated at frequent intervals. It was then placed in the ice-box overnight. In the morning, the mixture was centrifugalized and the supernatant fluid removed. Agglutination tests were set up, using the same dilutions as in the original agglutination tests. Controls were carried out with unabsorbed serum and normal rabbit's serum. All the tubes were placed in the water bath at 56 deg. C. for two hours. After standing in the ice-box overnight readings were made.

Complete absorption occurred in practically every case where there had been complete agglutination (Table 8). In the group containing

TABLE 8

Absorption Tests

Amount of Agglutination of Streptococcal Strains with Serums of 11 Immunized Rabbits before and after Absorption of Agglutinins

Strain	RB-19		RB-24		RB-9		RB-7		RB-25		RJ-36		RB-22		RB-16		RJ-34		RJ-17		RB-26	
	Un.	A.	Un.	A.	Un.	A.	Un.	A.	Un.	A.	Un.	A.	Un.	A.	Un.	A.	Un.	A.	Un.	A.	Un.	A.
RB-19	4	0	4	0	4	0	4	0	4	0												
RB-24	4	0	4	0							4	0										
RB-9	4	0			4	0					4	0										
RB-9(2)	4	0																				
RB-14	4	0																				
RB-18	4	0																				
RB-13	4	0																				
RB-7	4	0					4	0														
RB-25	4	0							4	0	4	0										
RJ-25	4	0							4	0												
RB-20	3	1											4	0								
RB-31	2	2													3	3	4	0				
RB-5	2	2									4	1			4	0					4	0
RB-21	0	4													4	0						
RJ-36											4	0							4	2		
RB-32															4	0						
RJ-16															4	0						
RB-15															4	0						
RB-29															4	0						
RB-27													4	0								
RB-2													4	2								
RB-10													4	0								
RB-23													4	0								
RB-22													4	0								
RJ-34																	4	0				
RB-34																	3	0				
RB-8																	4	0	4	2		
RB-28																	4	1				
RB-26																					4	0
RB-17																			4	0		
S. B. E.																			3	1		

In the absorption tests, the following dilutions of serums were used for the agglutination reactions; 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, and 1:2560. 4 = complete agglutination with sedimentation; 3 = agglutination and sedimentation with slight turbidity of the broth: 2 = agglutination without sediment; 1 = slight agglutination; 0 = no agglutination.

Un. = Unabsorbed serum. A. = Absorbed serum.

ten homogeneous strains, the agglutinins in the homologous immune serums were completely absorbed in every instance. This establishes the identity of these strains. The smaller groups indicated in the agglutination table can also be identified in the absorption table. (See absorption reactions with immune serums, RB-22 and RB-16.)

Relation of Streptococci Recovered from the Blood to Streptococci Isolated from the Joints

In 7 rheumatic fever patients who were subjected to joint cultures, 5 yielded a streptococcus, in every instance of the alpha or viridans type. In 4 of these 5 patients, streptococci culturally identical with those recovered from the joints were recovered from the blood cultures. Unfortunately one of the bloodstream streptococci was lost in subcultures. In the remaining three cases in which streptococci were isolated from both the blood and the joint, agglutination and absorption tests have showed them to be biologically identical (Tables 7 and 8). It appears from these findings that the joints in rheumatic fever are actually infected, and that the streptococcus responsible for the infection is the same strain that circulates in the blood.

DISCUSSION

In reviewing the literature dealing with the bacteriology of rheumatic fever, it has been pointed out that the results obtained by different investigators who have cultured the blood and the joints in this disease have varied from entirely negative results to a high percentage of positive results. It is significant, however, that when the results have been positive, the organism isolated has almost invariably been some form of streptococcus. That the problem is one almost entirely of technic is evident from a comparison of our own results in the Spring of 1928 with our results in the Spring of 1929. In 1928 the percentage of blood cultures positive for streptococci was 31 per cent. In the 1929 series, the percentage of positive streptococcus blood cultures was 83.9 per cent. If one goes still further and asks: What are the essential factors in the successful cultivation of streptococci from the blood and joints in rheumatic fever, the answer is not easy. Most important of all are technical

competence and experience, obtained preferably by work with streptococci. The first generation of these micro-organisms on artificial media is cultivated with great difficulty. Something is present in the medium which retards growth to a marked degree. In blood agar plates deep colonies are easily overlooked with the naked eye, and much help will be derived from a magnifying lens. As for the culture medium itself, an accurate titration of the pH. to 7.6 is important. Just how much of a factor is the particular beef-heart, tap water or peptone employed we are not prepared to say.

The organisms which have been isolated from the blood and joints in this series of cases appear to differ in no respect from those recovered by previous investigators. All but two strains have been classified as alpha streptococci (Str. viridans); one strain was classified as a beta streptococcus (Str. hemolyticus); one strain had no effect whatever on blood agar and was classified as a gamma streptococcus (Str. anhemolyticus). The amount of methemoglobin produced by the alpha streptococci varied considerably with the strain, some strains producing only a small amount. Even in these latter strains, however, a slight degree of hemolysis was produced on blood agar plates after 48 hours of growth, a characteristic which prevented our confusing them with gamma streptococci.

In this connection, a word should be added as to the bearing of the results herein reported on the work of Small (18) and of Birkhaug (21). Both of these investigators had their interest in the indifferent or gamma streptococci first aroused by the recovery of such an organism from the bloodstream of a patient with rheumatic fever. Small called his organism the "streptococcus cardioarthritidis;" Birkhaug referred to his strain as a "non-methemoglobin-forming" streptococcus. The evidence presented by these investigators in favor of the indifferent streptococcus as the exciting agent in rheumatic fever was based not so much on the successful recovery of this organism from the blood, joints and other lesions of rheumatic fever as it was on the frequent cultivation of gamma streptococci from the tonsils, throat and feces of patients with the disease. Hitchcock (22), however, has recently shown that indifferent streptococci are as frequent inhabitants of the throats of nonrheumatics as of rheumatic individuals. In our experience, as well as in that of other investigators who

have cultivated streptococci from the lesions of rheumatic fever, the streptococcus viridans plays a much more important rôle than the indifferent streptococcus.   It is true that a number of the strains which we have classified as alpha streptococci might have been placed in the gamma group by other investigators.   The criteria which we have employed compelled us to assign these organisms to the alpha group. The possibility, of course, of mutations from one form of streptococcus into the other must not be overlooked.

It appears from this and previous studies, then, that various types of streptococci can act as excitants in rheumatic fever.   Even the hemolytic streptococcus occasionally induces the symptoms and lesions of the disease.   (See case B-375 in this series and one case in Clawson's series.)   38 strains of streptococcus viridans recovered from our series of patients with rheumatic fever have been found by agglutination and absorption tests to fall into a number of biological groups.   The incidence and significance of these various groups will have to be determined by further study.

In our previously published studies on the bacteriology of the blood and joints in chronic infectious arthritis (20), it was shown by appropriate cultural and agglutination tests that the strains of streptococci isolated from affected joints were in each case culturally and biologically identical with the strain isolated from the patient's blood.   The same holds true with respect to the streptococci obtained from the blood and joints in rheumatic fever.   The serum of rabbits immunized against a streptococcus of blood culture origin has in every instance cross-agglutinated the streptococcus obtained by joint puncture in the same patient, and the degree of agglutination has been practically the same for the two strains.   .

The demonstration of streptococci in the blood and joints of a high percentage of patients with acute rheumatic fever makes it possible to draw certain analogies between this disease and other forms of arthritis, particularly gonococcal arthritis and infectious arthritis. In both these latter infections, a primary focus acts as a nidus for pathogenic bacteria which under certain circumstances break into the bloodstream and become localized in the joints where they set up metastatic infections.   It has long been known that such a sequence of events takes place in gonococcal arthritis.   In a recent study on

the rôle of the streptococcus in chronic infectious arthritis (20), we have presented evidence which goes far to show that a similar mechanism is at work in chronic infectious arthritis. From the findings reported in the present investigation it appears that rheumatic fever, at least in its acute stage, is a bacteremia and that streptococci circulate in the bloodstream and establish multiple secondary foci of infection in and about the joints.

During the last few years the rôle of allergy in the pathogenesis of rheumatic fever has been studied by Swift, Birkhaug, Zinsser and others, and much that is new and interesting has been added to our knowledge of this disease. Undoubtedly allergy plays an important part in rheumatic fever, just as it does in tuberculosis, syphilis and other infections. It is highly probable that patients infected with the rheumatic fever organism do not develop joint manifestations until a certain degree of susceptibility to this organism has been attained. Swift (23) points out, for example, that patients with rheumatic fever show a marked tissue reaction to streptococcus antigen, whereas patients with subacute bacterial endocarditis do not show the same state of reactivity to streptococci. During the hypersensitive state joint symptoms develop. On the other hand, in bacterial endocarditis, tissue immunity has been acquired and acute joint manifestations are rarely seen. Important, however, as these allergic phenomena are to a proper understanding of rheumatic fever, the fact must not be lost sight of that the demonstration of streptococci in the blood and in the joints of rheumatic fever patients provides convincing evidence that acute rheumatic fever is a bacteremia and that the joint manifestations are true infections differing in no essential respect from the metastatic joint manifestations observed in other bacteremias. Allergy undoubtedly exercises an important influence on the clinical manifestations of this infection. In both rheumatic fever and in bacterial endocarditis green streptococci are circulating in the bloodstream. In the former disease many joints become infected. In the latter disease, though the bloodstream usually contains more streptococci than are encountered in rheumatic fever, the joints remain free from infection. It is reasonable to suppose, therefore, that in rheumatic fever the patient's tissue is allergic to streptococci, while in infectious endocarditis the tissues

are immune to these organisms. A state of allergy toward the streptococcus, however, will probably not in itself induce the lesions or joint manifestations of rheumatic fever without the concomitant presence of streptococci. It is significant that vascular lesions and joint manifestations closely resembling those of rheumatic fever in man have been produced experimentally by several investigators by injecting streptococci of rheumatic origin into the ear veins of rabbits. So far, however, no one has produced the lesions or the joint manifestations of rheumatic fever in animals by the injection of streptococcus allergens.

SUMMARY AND CONCLUSIONS

1. During the Spring of 1928, 29 patients with acute rheumatic fever were subjected to blood cultures, of whom 9, or 31 per cent, yielded a streptococcus. During the Spring of 1929, 31 patients with acute rheumatic fever were studied by blood cultures, of whom 26, or 83.9 per cent, yielded a streptococcus. The higher percentage of positive cultures in the 1929 series appears to have been due to improved cultural methods.

2. Of the 35 strains of streptococci recovered from blood cultures, 33 have been classified as alpha streptococci (Str. viridans); one as a beta streptococcus (Str. hemolyticus); and one a gamma streptococcus (Str. anhemolyticus). Some of the viridans strains produced very little green on blood media.

3. Agglutination and absorption tests indicate that the strains of streptococcus viridans recovered from the blood of patients with rheumatic fever show a tendency to fall into specific biological groups.

4. In 7 patients with rheumatic fever who were subjected to cultures from affected joints, 5, or 71.4 per cent, yielded a streptococcus viridans. In 3 patients in whom green streptococci were recovered from both the blood and joint, agglutination and absorption tests proved the identity of the strains isolated from the two sources.

5. These findings corroborate those of previous investigators and make it difficult to escape the conclusion that rheumatic fever is a streptococcal infection usually of the *alpha* or *viridans* type.

6. The pathogenesis of rheumatic fever in respect to the joint lesions appears to be analogous to that of infectious arthritis and

gonococcal arthritis. Bacterial allergy probably influences the clinical picture in all three conditions, but in each instance the joint manifestations are primarily dependent upon localization of bacteria in the joint, with subsequent infection.

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NASOPHARYNGEAL FLORA IN HEALTH AND DURING RESPIRATORY DISEASE IN ISOLATED COMMUNITIES IN ALABAMA AND LABRADOR

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A report on the bacteriology of the nasopharynx in two isolated communities is given in this paper. The first community studied was in southern Alabama near the Gulf of Mexico, the second in the interior of Labrador. What was regarded as the normal flora was determined in each place. During the course of the studies, epidemics of nasopharyngitis or colds occurred in Alabama and of tracheitis in Labrador. These outbreaks were also studied bacteriologically.

The report embodied in this paper belongs naturally to the series of reports already published by Jordan, Park, Bloomfield, Noble and others on the nasopharyngeal flora in health and disease. The earlier studies were carried out in large population centers in which the environmental factors are numerous and uncontrollable.

In an effort to minimize the extraneous factors, we undertook the study of isolated communities, where life is simple, outside contacts infrequent, and environmental factors more accurately determinable and controllable.

Methods

The method pursued was that of taking nasopharyngeal swabs by means of West tubes. The swabs were drawn from the glass tube with sterile forceps and placed in tubes containing 5 per cent blood hormone broth. When cultures were taken at a distance from the laboratory they were placed immediately in a water-jacketed container at 37°C. for transport. Usually the tubes were incubated at 37°C. for three hours in order to secure uniform incubation periods independent of transportation times and to suppress the Gram-negative cocci as well as to promote growth of pneumococci and Pfeiffer bacilli if present in small numbers.

After the preliminary incubation, the swabs were whipped vigorously in the medium and discarded. A loopful of blood broth was streaked on 3 to 5 per cent blood agar plates which were incubated for 36 to 48 hours. Representative type colonies were fished from the plates and the percentage of each type of colony present was estimated. The identification procedures were those in common use.

The liquid medium employed consisted of hormone broth (Fisk and Burky(1)). In Alabama beef infusion and in Labrador sheep hormone broth agar were also used as basic media. Hormone broth enriched with 1 per cent sheep serum and 1 per cent sugar with brom cresol purple as indicator served for satisfactory fermentation tests. In Labrador the pneumococci grew slightly or not at all in sheep hormone broth, but the addition of 1 drop of sterile sheep serum to 5 cc. of the broth provided the necessary accessory substance to produce luxuriant growth.

Rabbit, sheep and horse blood agar were employed at various times, the most satisfactory being rabbit blood agar medium. All organisms commonly found in the nasopharynx, particularly the Pfeiffer bacilli and the pneumococci, grow well on it. The pneumococcus colony is more characteristic than on the other kinds of blood media. Horse blood is also satisfactory but Pfeiffer bacilli grow less well and pneumococci form less characteristic colonies. Sheep blood is not satisfactory because the Pfeiffer bacilli grow poorly on it.

Places and Groups Studied

Alabama

The first study was conducted in southern Alabama from November 1927 to May 1928. The community selected consisted of about twenty-five families living in small clearings in the forest along Patsiliga River and named appropriately Happy Hollow. The cultures were taken from the school children. This district is well isolated from the trading centers of Andalusia and Greensville both by distance (20 miles) and the poor condition of the roads. The visits of the male adults to the more populous centers are confined to occasional trading visits and semiannual attendance at court, when the women sometimes accompany them. The children rarely go to town. The school teacher makes monthly visits to town to attend a teachers' conference. Few outsiders visit the region. The economic status of . the people is poor.

The Happy Hollow school is a well-ventilated one room frame building, heated when necessary by a wood-burning stove. It seats without crowding its twenty-three pupils whose ages range from six

to sixteen. All children and the teacher live within three miles of the school. The teacher, a capable woman, supplied much of the requested information.

Seven sets of cultures were taken at this school at irregular intervals. They have been divided on the basis of clinical condition into the following groups:

(*1*) *Period of Normal Health.* In November and December two sets of cultures were taken when all members of the group were in normal health.

(*2*) *Period of Acute Colds.* In January there was a marked fall in temperature to below freezing. This period of cold (unusual for this subtropical region) continued for about two weeks, after which there was a considerable rise in temperature. At about this time the teacher returned from a week-end visit to Andalusia, where, according to her statements, she acquired a cold which was so severe that she likened it to influenza, which she had had during the epidemic period of 1918. The first cultures in this period were obtained about three days after her return. At this time four children also had colds that had developed during the preceding few days. One week later every person in the school had a cold, and another set of cultures was taken. One week later another set was taken. At this time a few individuals had recovered, but the great majority had coughs with varying amounts of nasal discharge.

(*3*) *Period of Recovery.* One set of cultures was obtained during March, one month after the cold epidemic had reached its height. At this time every one seemed normal. In April another set of cultures was secured, at which time every one complained of a slight cold. The teacher expressed the opinion that these colds were chronic and had existed in slight or moderate degree since the epidemic. For this reason this group of cultures has been given the foregoing title.

The bacteriological results have been summarized in Table I and charts I to VII and will be discussed in conjunction with the findings in Labrador.

Labrador

The second study was carried out during July, August, and September at Northwest River, Labrador, a Hudson Bay Company

trading post of about 150 inhabitants, situated at the mouth of North-west River, where it flows into Lake Melville. The only contact that this community has with the outside world is through Rigolet, 100 miles away on the sea coast, where the mail boat calls about once in three weeks during the summer months. Once during the winter a driver with his dog team brings the mail in over the ice.

The climatic conditions at Northwest River are delightful during the summer months. They resemble most nearly those of Alabama during the month of November. The temperature rarely exceeds 90°F. and only in September did the minimum temperature fall to freezing. The humidity remains consistently low except just before heavy rain-falls.

For purposes of comparison and discussion the people at Northwest River were divided into the following groups.

Transient. This group was small (about fifteen) and was composed of summer visitors to Northwest River who came in with the first mail boat at the end of June. The majority of this group were Americans and English who came directly from their home countries. Only "normal" cultures were obtained from this group.

Permanent. About ten of this group are whites of American and European extraction who are connected either with the Grenfell Mission station or the Hudson Bay Company and who spent the preceding winter at Northwest River. Only "normal" cultures were secured from this group.

The remainder, and the great majority, of this group is composed of so-called natives. They are a mixture of white and Indian with an occasional trace of Esquimo blood. Their main occupation is trapping, and their economic status is poor. As a result their diet is largely limited to bread, molasses, fish—fresh or salt—and salt meat. Fresh vegetables are practically unknown. The health of the community, despite the pioneer conditions of living, is good. Tuberculosis of the acute fulminating type is the outstanding acute disease. Pneumonia is rare, and when it does occur is mild. In the influenza epidemic of 1918, the mortality was high. A minor epidemic of influenza occurred in March, 1928, with no fatalities. Childhood diseases such as measles, mumps, whooping cough, scarlet fever, and diphtheria are practically unknown.

Indian. During the early summer the population of Northwest River was increased by the appearance of about 150 Montaignais Indians, who came to trade with the Hudson Bay Company and receive the ministrations of the Roman Catholic priest. The contact of the Indians with the remainder of the population was slight, and for this reason they have been grouped separately.

The economic status of the Indians is even poorer than that of the natives.

They are a nomadic race living in tents, and their diet is even more restricted than that of the natives. They are subject to about the same diseases as the natives.

Colds. Shortly after the arrival of the summer visitors a few cases of colds of moderate severity developed among the native and Indian populations. Only four of the natives were considered as having "bad colds." The Indians spoke no English and visited the laboratory for amusement with a variety of complaints. Thus the classification of *"normal"* and *"colds"* in this group is somewhat confused. Those listed as *normals* were persons with no complaints referable to the respiratory system, but it is difficult to be sure whether or not all in the cold group actually had colds.

Tracheitis and Contacts. In the latter part of August, after the departure of the Indians, a definite epidemic of tracheitis developed which affected practically every one in the community in greater or lesser degree, with the exception of the white Grenfell Mission staff and ourselves. Cultures were obtained from the native population only, and were divided into two groups; those with the disease at the time of examination and those listed as "contacts," who were exposed intimately to the disease or who developed it within a few days after the cultures were taken. Because the appearance of this disease was coincident with the arrival of outsiders from a place where so-called influenza was epidemic, and coincident also with the appearance of Pfeiffer bacilli in the community, the bacteriological findings will be discussed when the Pfeiffer bacilli are considered. They are summarized in Table I and Graphs I to VII.

Discussion of Findings

Groups of Organisms. The bacteria found have been classified under the headings seen in the table and the graphs. None of these groups requires any special mention except the *intermediates*, composed of Gram-positive diplococci or streptococci, producing green or viridans hemolysis on blood agar plates. The intermediates do not, however, fall definitely either into the green streptococcus or the pneumococcus groups but occupy a midway position, if the classification is based on colony formation, morphology, inulin fermentation, and bile solubility. Since the classification of these organisms is indefinite and since they

are not characteristic pneumococci, they have been recorded separately, and grouped also with the typical green streptococci.

In recording the occurrence of the various types of organisms on the blood agar plates, an estimate of the percentage of each type present was made. The estimates were recorded in multiples of five, and the least estimate was five per cent. The percentage figures for each type of organism in any group of individuals were then added together and divided by the number of examinations made. The resulting figure has been termed the "bacterial group incidence" and represents a figure which would presumably be secured, theoretically at least, by pooling all of the nasopharyngeal swabs from one group of individuals and streaking the pooled emulsion upon one gigantic blood agar plate. Since the persons in each group come into close contact, and their states of health are so similar, the bacterial group incidence figures may be assumed to represent the relative numbers of bacteria present in, as it were, a community throat.

Using this method a figure is obtained, the accuracy of which is undoubtedly open to criticism on technical grounds. When preliminary incubation was used, some groups of organisms were suppressed and others favored, besides which variations in plating technique, and in estimation of percentages of organisms must occur, as well as other technical errors. However, it is believed that these variations and errors are more or less constant and tend to balance one another and that the percentage figure obtained by the method cited above represents a fairly true mean of what may be found in one group of individuals, and is of value in comparing the findings in one group with that in another or the same group in a different state of health, provided that minor differences in figures are disregarded. Therefore in discussing the "bacterial group incidence" only such differences as have been found statistically significant will be considered.

The bacterial group incidence data are imperfect in that they do not show the distribution of organisms in individual throats. Blood agar plates from some individuals in a community showed large numbers of a given organism; others showed few or none. In order to indicate this individual variation we have computed the mean and the standard deviation for each organism and for each series of persons studied. See Table I.

TABLE I

Bacterial Group Incidence of Nasopharyngeal Flora: Normal, Colds, and Trachcitis

The mean with standard deviation is given in each instance

	No. exam.	Gram neg. cocci %	Staphylo-cocci %	Influenza %	Streptococci %			Intermedi-ate %	Green and intermed. %	Pneumo-cocci %	Others %
					Hemolytic	Indifferent	Green				
Normal											
Alabama											
1st Exam.	17	45 ± 6.4	2 ± 1.0	0	0	0	49 ± 5.7	0	49 ± 5.7	0	3 ± 2.0
2nd "	17	49 ± 8.8	2 ± 0.6	12 ± 6.3	0	0	24 ± 7.5	8 ± 4.9	32 ± 7.5	0	6 ± 2.6
Labrador											
Transient	19	16 ± 6.6	2 ± 0.8	5 ± 2.4	1 ± 1.0	12 ± 5.3	18 ± 6.4	22 ± 8.1	40 ± 8.1	18 ± 7.9	5 ± 3.3
Permanent	25	10 ± 3.2	2 ± 0.7	2 ± 1.4	0	13 ± 3.9	37 ± 7.2	7 ± 4.8	44 ± 7.3	22 ± 6.5	6 ± 2.7
Indian	21	12 ± 2.5	2 ± 0.7	3 ± 6.9	1 ± 1.0	11 ± 3.3	18 ± 4.6	3 ± 2.2	21 ± 4.7	46 ± 7.3	3 ± 1.0
Colds											
Alabama-Epidemic											
1st Exam.	14	15 ± 3.1	7 ± 2.5	21 ± 5.7	6 ± 4.4	10 ± 4.6	16 ± 5.4	19 ± 5.4	35 ± 4.0	3 ± 1.7	3 ± 5.1
2nd "	19	14 ± 2.4	7 ± 2.4	18 ± 4.4	6 ± 3.0	1 ± 1.0	30 ± 6.2	1 ± 1.0	31 ± 6.2	19 ± 6.0	3 ± 1.7
3rd "	15	14 ± 4.0	3 ± 2.5	28 ± 6.1	7 ± 3.7	0	22 ± 5.5	23 ± 8.3	45 ± 5.4	2 ± 1.7	1 ± 1.3
Recovery											
1st Exam.	15	26 ± 5.7	2 ± 1.7	41 ± 7.7	6 ± 3.3	4 ± 2.7	17 ± 5.1	4 ± 2.7	21 ± 2.7	0	0
2nd "	19	16 ± 5.2	7 ± 2.9	6 ± 2.5	0	4 ± 1.7	44 ± 7.5	12 ± 6.3	56 ± 7.3	1 ± 0.5	—
Labrador Sporadic											
Permanent	23	21 ± 5.3	2 ± 1.0	5 ± 3.0	0	15 ± 4.6	16 ± 4.2	10 ± 4.5	26 ± 5.5	23 ± 6.9	8 ± 3.9
Indian	19	12 ± 2.5	4 ± 1.3	3 ± 2.6	0	3 ± 1.4	19 ± 6.6	6 ± 4.4	25 ± 3.3	45 ± 6.5	9 ± 3.5
Trachcitis											
Labrador											
Contacts	16	15 ± 4.8	4 ± 3.7	20 ± 7.5	0	3 ± 1.7	17 ± 6.9	14 ± 6.5	31 ± 7.7	21 ± 7.2	5 ± 3.1
Trachcitis	35	15 ± 2.6	3 ± 2.6	12 ± 3.7	3 ± 1.0	17 ± 4.1	22 ± 4.4	10 ± 3.5	32 ± 5.1	16 ± 3.3	3 ± 1

The incidence of any given organism in individual throats is shown in the graphs. For example, in Graph I—Gram-negative cocci—we have indicated the total percentage of persons in each series harboring this type of organism. We then subdivided the positive cases into four groups on the basis of the proportional number of this particular type of organism found on the individual blood agar plates. Thus in the series of Alabama normals, on first examination, 100% of the persons showed gram-negative cocci, over one fourth (27%) of these persons had from 1–25% of gram-negative cocci in relation to all other organisms, 37 per cent of the persons showed 26 to 50% gram-negative cocci, 22% showed 51 to 75% of these organisms, and in the remaining 14% of the positive cases from 75 to 100% of the total flora were gram-negative cocci. See Graph I.

The findings for the "staphylococci," "hemolytic streptococci," and "others" have not been represented graphically because of the relatively small numbers of these organisms found.

During the course of this study various individuals have been examined for the presence of filter-passing anaerobic organisms. The results will be presented in another report.

Gram-Negative Cocci. (Table I, Chart I). During the study considerable variation in colony formation, pigment production, and action on sugar media of the same strains of organisms in this group have been noted. Recently Wilson and Smith (2) have reported at length on such variations and concluded that the members vary from time to time, and they suggest that for the present, at least, the members, with the exception of the meningococci, be classified as belonging to a large variable group and no attempt be made to divide them into species on the basis of the above-mentioned factors. For this reason we have made no effort to divide the members of this type into species. We found no meningococci.

The bacterial group incidence is fairly constant in all of the persons studied except the Alabama normals. The relatively large numbers of gram-negative cocci present in the nasopharynx of the normal persons studied in Alabama may be due to the fact that blood agar plates were inoculated directly with the swabs without giving them a preliminary blood broth incubation. The individual incidence is about the same in all the persons studied. During the periods of acute infection

there is a tendency to suppression of the organisms.   This fact suggests that the Gram-negative cocci play no part in the acute respiratory infections.

Staphylococci.   (Table I).   The types found were *S. albus* and *S. aureus* and in Labrador an occasional *S. citreus*.   A few of the *S. albus* and *S. aureus* strains were hemolytic.

THE INCIDENCE OF GRAM NEGATIVE COCCI IN THE NASOPHARYNGEAL FLORA

			INDIVIDUAL INCIDENCE	BACTERIAL GROUP INCIDENCE
			Per cent of individuals having varying intensities of Gram Negative Cocci 10 20 30 40 50 60 70 80 90	Per cent 10 20 30 40 60
NORMALS	Alabama	1st Exam.		
		2nd Exam.		
	Labrador	Transient		
		Permanent		
		Indian		
COLDS	Alabama (Acute)	1st Exam.		
		2nd Exam.		
		3rd Exam.		
	Alabama (Recovery)	1st Exam.		
		2nd Exam.		
	Labrador (Sporadic)	Permanent		
		Indian		
TRACHEITIS	Labrador	Contacts		
		Tracheitis		

□ 1 – 25%   ▨ 26 – 50%   ▧ 51 – 75%   ■ 76 – 100%

Per cent of Gram Negative Cocci in relation to all other organisms in the nasopharynx

CHART I

The bacterial group incidence of these organisms is low, in normal persons small numbers being found in about half of the individuals examined.   During infection the incidence rose slightly, though there was not a constant distribution.   A few individuals harbored large numbers and in the remainder staphylococci were entirely absent. During the recovery period in Alabama, almost pure cultures of staphlococci were recovered from certain individuals with chronic colds.

Pfeiffer Bacilli.   (Table I, Chart II).   This group of organisms is

composed of Gram-negative rods requiring some accessory factors for growth and includes the para and hemolytic strains.

The bacterial group incidence in Alabama increased with the appearance of the cold epidemic as it did in Labrador with the appearance of the tracheitis epidemic. In Alabama, however, the highest incidence was found one month after the epidemic had reached its peak. In Labrador it was not possible to determine whether or not the

THE INCIDENCE OF INFLUENZA BACILLI IN THE NASOPHARYNGEAL FLORA

CHART II

highest incidence occurred sometime after the epidemic abated, because of the discontinuance of this study just at the end of the epidemic. In general, the individual incidence rose parallel with the bacterial group incidence.

During normal periods, particularly in Labrador, the majority of the Pfeiffer strains which were recovered were either para or hemolytic in type. During periods of infection there appeared large

numbers of true Pfeiffer bacilli, e.g. non-hemolytic strains requiring both v and x substance for their growth. Most of them were of the indol-forming type. The numbers of para strains recovered remained about constant at all times, while during epidemics there was a marked increase in the number of strains of true Pfeiffer bacilli recovered. This phenomenon was noted by Park (3) and his co-workers.

In Labrador the association of Pfeiffer bacilli with the outbreak of the tracheitis epidemic was so conspicuous that the following detailed story of the epidemic is included at this point.

During the latter part of July an epidemic of acute respiratory disease appeared in Cartwright and other coastal settlements over one hundred miles away. The cases were characterized by acute onset, prostration, bone aches, and fever of about five days' duration. Many patients remained prostrated for two or three weeks after other symptoms disappeared. The cases were diagnosed as "influenza" by the three physicians of the Mission hospitals on the coast.

The mail boat arrived in Northwest River from the coast on August 8, bringing from Cartwright a child of nine years, who probably had just recovered from an attack of "influenza." Her contacts after arrival in Northwest River were few, and apparently no secondary case occurred from contact with this child. On August 9, two natives returned from a trip to the coast. One had caught a slight cold in Indian Harbor following association with deep sea fishermen. The man with the cold lived on the south bank of Northwest River and had little contact with the community. The day following his return, his wife had a chill and headache and came to the laboratory on the 15th complaining of a cold. Her only symptom was sore throat, and cultures showed no Pfeiffer bacilli.

On August 15 an English clergyman arrived from Cartwright and brought the first news of the influenza epidemic. He had been closely associated with it but had not been ill. Cultures of his nasopharynx taken one hour after arrival showed about ten per cent Pfeiffer bacilli. Otherwise, the flora was similar to that of the local inhabitants. During the next two days he visited all of the houses in the village and on August 16 held a communion service which was attended by practically every one in the village.

The results assembled under "contacts" were obtained during the next few days.

On August 18 the native who returned in good health from Indian Harbor awoke with a headache and a tickling senation in the throat, localized by him as just below the larynx, and accompanied by a dry unproductive cough. Two days later his wife developed an illness with the same onset. The infection spread rapidly, and by September 4 practically every one in the village had had a similar attack. Individuals reacted differently, but general tendencies were noticeable. In children, the irritation and cough continued for several weeks and were much

worse at night.   The younger adults who had the attack early in the outbreak recovered rather promptly with few or no other symptoms.   Those who were attacked later in the outbreak had the usual onset, but two or three days later had what may be best described as violent head colds with prostration and fever of several days' duration.   The older adults were entirely unaffected.   Upon our departure on September 17 numerous cases of the head colds persisted, but in general the epidemic could be said to be subsiding.

In summarizing the facts of this outbreak it can be said that following the arrival of a potential carrier or carriers of "influenza organisms" directly or indirectly from Cartwright, where there had been an influenza epidemic, there occurred an epidemic of tracheitis at Northwest River.   The most probable carrier of the organism was the clergyman who harbored Pfeiffer bacilli in his nasopharynx on arrival. Coincident with the epidemic, (in Northwest River), there was a statistically significant increase in the bacterial group incidence and the individual incidence of true Pfeiffer bacilli in the community. See Chart II and Table I.)

Hemolytic Streptococci.   The types of organisms found in Alabama were *St. pyogenes, St. anginosus,* and *St. infrequens* (according to Holman's classification).   The only type found in Labrador was *St. pyogenes,* of a low virulence for mice.

Few persons harbored members of this group and in these persons the numbers of organisms present were small.   In Alabama these organisms were found only during the cold epidemic, and in Labrador, with one exception, only at the time of the tracheitis epidemic.   In Labrador the streptococci were found in the nasopharynx of individuals who had impetigo, from the lesions of which similar strains of *St. pyogenes* of low virulence were recovered.   These persons were all examined one or more times before the onset of the epidemic, and the hemolytic streptococci were not found in the nasopharynx until after the outbreak.   These findings suggest that the hemolytic streptococci play the rôle of a secondary invader and resemble in this respect the staphylococci.

Indifferent Streptococci.   (Table I, Chart III).   In this group are included Gram-positive cocci, usually in short chains with a characteristic colony formation, that do not act upon blood and that almost invariably ferment inulin.   Upon lactose and salicin the action is variable.

This type of organism was not found during the normal period in Alabama. Early in the cold epidemic a few strains were found in an occasional individual and were then present in small numbers only. In Labrador, on the contrary, the organisms were present in moderate numbers and in the great majority of persons examined and constituted apparently part of the normal flora.

A small series of observations made upon the members of the transient group of persons at Northwest River indicates that the indifferent

CHART III

streptococcus was implanted in these persons after their arrival at Northwest River, and became a part of their normal flora. These persons were examined ten days after their arrival and showed no indifferent streptococci. Second and third examinations of these individuals were made later during the summer and with each examination increasing numbers of indifferent streptococci were recovered.

Green Streptococci, Intermediates, and Pneumococci. (Table I,

Charts IV, V, VI, VII). Because of the cultural relationships of these organisms they will be discussed together. The types of green streptococci and the order of frequency with which they were found were *St. salivarius, St. mitis, St. equinus, St. ignavus* and *St. fecalis*. The last was found rarely. *St. salivarius* was the most common while *St. equinus* and *St. ignavus* were found occasionally in each group.

The "intermediates" were organisms which resemble both pneumococci and *St. viridans*. Their fermentation reactions are, as a rule,

THE INCIDENCE OF GREEN STREPTOCOCCI IN THE NASOPHARYNGEAL FLORA

CHART IV

like those of *St. salivarius*, but in addition they fermented inulin. The colony formation and the morphology in smears was such as to suggest the pneumococcus. These strains were usually partially bile soluble. This group also includes strains that were typical green streptococci except that they were bile soluble. The outstanding type of this group showed characteristic green colonies and morphology, did not ferment inulin, and in broth grew in a mass at the bottom of the tube.

This mass when broken up did not emulsify smoothly, but formed large flakes which were bile soluble. The members of this group that were tested were not virulent for mice.

The organisms classified as pneumococci were typical in colony formation, morphology, inulin fermentation, and bile solubility. In Alabama, virulence tests were not made, but in Labrador a sufficient number were carried out to show that the organisms were comparatively avirulent to white mice.

CHART V

The green streptococci were obviously normal inhabitants of the nasopharynx. With the onset of an "infection" there was a diminution in numbers in the affected person with complete suppression in a few individuals. (See Chart IV.)

The intermediates were found in all groups, and in general, as they appeared, the green streptococci diminished. Whether this reciprocal

relation is due to errors in detecting colonies for identification and in estimating the numbers present, or whether the disappearance of the green streptococci is accompanied by a definite increase in the intermediates, was not determined definitely.

Examinations during the normal period in Alabama gave few or no intermediates. When the cold epidemic first appeared in Happy Hollow school the green streptococci were suppressed and the inter-

THE INCIDENCE OF GREEN STREPTOCOCCI AND INTERMEDIATES IN THE NASOPHARYNGEAL FLORA

CHART VI

mediates appeared in numbers equivalent to the missing green streptococci. At the height of the epidemic, the intermediates had disappeared while the green streptococci returned to approximately their previous numbers. Coincident with the disappearance of the intermediates there was a significant rise in the number of pneumococci found.

Pneumococcus. (See Chart VII.) In Alabama the pneumococcus

was not found during the normal period. Coincident with the appearance of the epidemic of colds, pneumococci were found in considerable numbers, the incidence being highest at the height of the epidemic. At the end of the epidemic the pneumococci disappeared.

In Labrador, on the other hand, pneumococci made up a large part of the normal flora. The transient population showed the lowest incidence, the trappers a relatively high incidence, and the Indians the

THE INCIDENCE OF PNEUMOCOCCI IN THE NASOPHARYNGEAL FLORA

CHART VII

highest of all. In fact, the predominating organism in the throats of the normal Indians was a pneumococcus, type IV, of low virulence to mice. No colds occurred among the transient group, but in those individuals from both Indian and permanent groups who had sporadic colds there occurred no increase in the incidence of pneumococci. The epidemic of tracheitis, which affected the permanent population after the nomadic Indians had left the Post, was not accompanied by an

increase in pneumococci.   If the Indians are considered as the group furthest removed from civilization it follows that the pneumococcus becomes increasingly a part of the normal flora in Labrador as civilization is left behind.   Yet pneumonia is rare in these regions both among the trappers and the Indians and when it occurs it is seldom fatal.

Other Organisms.   In this classification we have included a miscellany of types of organisms, none of which was found with any regularity or associated with any pathological condition.   The outstanding forms were diphtheroids, large Gram-negative rods, and strains of small Gram-positive cocci which produced no, or slight, traces of viridans hemolysis when grown in mass cultures on blood agar plates. Some of the latter strains slightly fermented dextrose, others fermented no sugar whatever.   In colony formation these cocci resembled the indifferent streptococci and would have been so classified but for the traces of hemolysis and their inactivity in sugar media.

Comparison of Findings with the Results of Other Investigations

In comparing the results of the present investigation with those obtained by others, several rather well-established facts have been confirmed.   The Gram-negative cocci, staphylococci, green streptococci, diphtheroids, and possibly also hemolytic streptococci are normal inhabitants of the nasopharynx.   In so far as statistical methods offer any proof, none of these groups of organisms was related to the two epidemics of colds which were studied.

The method of reporting results adopted by Park (3) and his co-workers is essentially the one used in the present study and although the technique used by him was more elaborate the results can be compared directly.   The general agreement in results leads to the belief that the discrepancies noted are real and not dependent upon variations in technique.   Table II compares our results with those of Park and his co-workers.

The chief differences between the New York data as given by Park and those from Alabama and Labrador are:

Normal Flora.   Pneumococci were more prevalent in New York than in Alabama, whereas in Labrador pneumococci were much more prevalent than in New York.   Pfeiffer bacilli were more prevalent in

TABLE II

Comparison of Nasopharyngeal Flora in New York (Park et al.) with Alabama and Labrador

		Gram-neg. cocci	Staphylococci	Pfeiffer bacilli	Hemolytic strep.	Indifferent strep.	Green Strep. & Intermediates	Pneumococci	Others	Remarks
Normal										
Bacterial Group Incidence	Alabama	47	2	6	0	0	40	0	5	Average of two normal examinations.
	Labrador	11	2	2	1	12	33	34	5	Average of Indian and Permanent group.
	New York	29	6	9	1	2	32	8	11	
Individual Incidence	Alabama	100	24	17	0	0	95	0	18	
	Labrador	89	33	14	5	58	80	60	33	
	New York	87	12	40	6	4	89	26	39	
Colds										
Bacterial Group Incidence	Alabama	14	7	18	6	1	31	19	3	Height of epidemic.
	Labrador (Sporadic colds)	17	3	4	0	9	26	34	7	Average of Indian and Permanent.
	Labrador (Tracheitis)	15	3	15	2	12	32	18	4	Average of Contacts and Tracheitis.
	New York	19	2	17	3	2	33	9	19	
Individual Incidence	Alabama	100	20—	75	10—	5	65	52	25—	
	Labrador (Sporadic colds)	87	20—	12	10—	30	65	63		
	Labrador (Tracheitis)	73	20—	45	1—	55	78	49	25—	
	New York	87	65	65	16	34	91	39	70	

New York than in Alabama or Labrador. Indifferent streptococci were more prevalent in Labrador than in New York. In Alabama these organisms were not found in normal throats. Hemolytic streptococci were not as prevalent in normal throats in Labrador or Alabama as in New York.

Colds and Tracheitis. An epidemic of colds in Alabama was accompanied by an increase in pneumococci and Pfeiffer bacilli. Sporadic colds in Labrador did not change the essential picture but an epidemic of tracheitis was accompnaied by an increase in the prevalence of Pfeiffer bacilli. In New York colds were accompanied by an increase in Pfeiffer bacilli.

Except for these rather striking differences, there is close parallelism between the nasopharyngeal flora in an isolated semitropical farming community in Alabama, a remote semi-arctic trading post in Labrador, and New York City.

SUMMARY

Studies of the bacterial flora of the nasopharynx were made in isolated communities in South Alabama and Labrador. The basic flora was determined in both communities. In Alabama an epidemic of common colds was studied. In Labrador cases of sporadic colds and an epidemic of tracheitis were studied:

Gram-negative cocci were found in nearly all normal individuals in moderate numbers. In pathological states there was a suppression of these organisms.

Staphylococci were found in small numbers in about half of the normal individuals. In pathological conditions they disappeared from most of those affected but were found in increased numbers in a few individuals.

Pfeiffer bacilli were absent or present only in small numbers in normal individuals. During the epidemic of colds in Alabama there was an increase in the number of strains recovered and an increase in the relative numbers of the bacilli in each throat. The highest prevalence was found one month after the epidemic had reached its height. In Labrador a similar increase was coincident with an epidemic of tracheitis. During normal periods the majority of the Pfeiffer strains were of the para non-indol-forming type. During

epidemic periods the strains recovered were largely true indol-forming *B. Pfeifferi.*

Hemolytic streptococci were rarely found in normals. During disease prevalence periods they appeared in a small number of persons.

In Alabama, indifferent streptococci resembled the hemolytic streptococci in their distribution. In Labrador they were found to be widely distributed in both health and disease and composed apparently a part of the normal flora.

Green streptococci were found to be widely distributed in fairly large numbers both in health and disease.

Intermediates, or organisms midway between green streptococci and pneumococci, were found in moderate numbers in each series of persons studied. Early in the Alabama epidemic they were present in large numbers in nearly all persons.

Pneumococci were not found in Alabama in normal individuals. The epidemic of colds in Alabama was accompanied by a marked increase in the incidence of these organisms. In Labrador pneumococci seemed to be part of the normal flora as they were generally distributed throughout the community, in many instances comprising a large proportion of the flora of an individual's throat. The Labrador strains of pneumococci were avirulent.

A variety of other organisms such as diphtheroids, Gram-negative rods, and Gram-positive cocci were found in small numbers in many individuals both in health and disease.

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DEVELOPMENT IN TISSUE CULTURES OF THE INTRACELLULAR CHANGES CHARACTERISTIC OF VACCINAL AND HERPETIC INFECTIONS

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PLATES 29 TO 31

In a preliminary note (1)* a method of obtaining in tissue cultures intracellular changes characteristic of vaccinal and herpetic infections was briefly described. The purpose of the present paper is to give a detailed report concerning this method and some of the results secured through its use.

In 1906, Aldershoff and Broers (3), in order to prove that Guarnieri bodies are not derived from leucocytes, inoculated rabbit corneas with vaccine virus, immediately removed them from the animals, and incubated them in sterile serum at 37°C. This method was unsuccessful, the corneas becoming contaminated with bacteria which in a liquid medium interfered with the work. Thereupon the workers altered their procedure and placed the inoculated corneas, suspended in physiological salt solution, in humid chambers. After incubation for different periods of time, the tissues were fixed, sectioned, stained, and examined for the presence of vaccine bodies. Guarnieri bodies were found in corneas treated in this manner.

Gins (4), in 1916, using the coverslip method for the cultivation of vaccine virus in corneal cells, obtained what he considered Guarnieri bodies. Later (5), however, he stated that the bodies observed by him in 1916 were probably not vaccine bodies.

The majority of workers, including Steinhardt, Israeli, and Lambert (6), Harde (7), and Haagen (8), who searched for Guarnieri bodies in their tissue

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* Shortly after this note was sent in for publication, one of the authors (Rivers) received a letter from Dr. C. H. Andrewes regarding the appearance of inclusions in cultures of testicular tissue infected with Virus III. His observations and our findings were made independently and approximately simultaneously. A preliminary note (2) of his work appeared in *Brit. Jour. Exp. Path.*, 1929, 10, 188.

cultures infected with vaccine virus, failed to find them. · It also appears that no investigator has observed nuclear inclusions in tissue cultures infected with herpetic virus. Andrewes (2), however, in a recent report, has described the appearance of characteristic nuclear changes in testicular tissue infected with Virus III and cultivated *in vitro*.

Intracellular changes, "inclusion bodies," either cytoplasmic or nuclear, are characteristically associated with certain virus infections as they occur within the living host. These alterations, although their exact nature has not as yet been determined, are frequently used for diagnostic purposes. In spite of the fact that inclusion bodies occur in infected cells within the host, no observer, until recently (1, 2) has found them in tissues infected and grown *in vitro*. Inasmuch as virus infections, virus immunity, and even the nature of viruses themselves are poorly understood, it seemed that, if it were possible to devise a means of securing visible evidences of virus activity in tissue cultures, definite information might be found concerning many obscure points in this field of work. After a number of unsuccessful attempts, the following method of regularly obtaining Guarnieri bodies and herpetic nuclear inclusions in tissue cultures was evolved.

Materials

Viruses.—Vaccine virus and the virus of herpes simplex were employed because the rabbit is equally susceptible to both. Furthermore, the vaccine virus produces cytoplasmic changes while that of herpes induces nuclear alterations. Thus, one virus served as a control on the other.
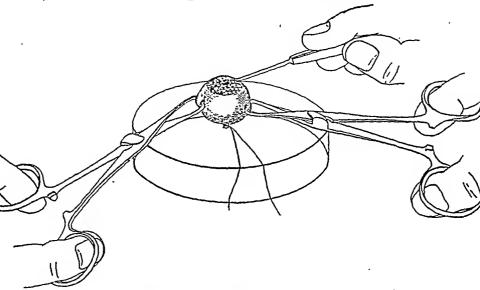
Levaditi's neurovaccine propagated in the testicles of rabbits, and Noguchi's testicular virus, were the strains of vaccine virus used. The herpetic virus chosen for the work was the H. F. strain isolated by Flexner and Amoss. In all experiments save one, freshly prepared virus emulsions without glycerol were employed. For constant results, one must conduct the experiments with fresh potent virus to which no preservative has been added.

Tissues.—For this work, rabbit corneal tissue was chosen because of the following facts: 1) rabbit corneal epithelium is equally susceptible to the viruses of vaccinia and herpes simplex; 2) Guarnieri bodies and herpetic inclusion in cells of this tissue have been extensively investigated; 3) in the past, corneal cells have frequently been used for the *in vitro* cultivation of vaccine virus; 4) many studies concerning vaccinal and herpetic immunity within the animal have been conducted in the cornea of rabbits.

Procedure

A rabbit, under light ether anaesthesia, was exsanguinated from the heart by means of a syringe into which 1 cc. of a 1–1,000 heparin solution for each 10 cc. of blood had been previously drawn.   Then, to obtain a tissue extract, the spleen was excised, minced, added to a small amount of Ringer's solution, and centrifuged.   Finally, both eyes were enucleated intact, after stout silk ligatures had been placed around the nerve and large vessels.

The eyes, fixed in the grip of special forceps (Text-fig. 1) and suspended over a large Petri dish, were thoroughly washed with Ringer's solution to remove as many contaminating bacteria as was possible.   With a sterile cataract knife the

TEXT-FIG. 1. Manner in which corneas were scarified and infected with the viruses.
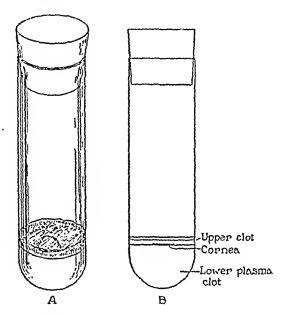
eyes to be used as controls were cross-hatched (Text-fig. 1) with closely spaced scarifications just deep enough to penetrate the epithelial layer.   Test eyes were treated in a similar manner with the exception that prior to each scarifying stroke the knife was dipped in a virus emulsion.   The cornea was then removed from each eye and divided into 4 to 8 pieces.   The pieces from test eyes were further inoculated by immersion in an emulsion of virus for 1 to 3 hours at 37°C.

In some experiments antemortem inoculations were made.   Following an instillation of a 2 per cent cocain solution, the corneas were scarified and inoculated in the usual manner.   1 or 2 hours later the animals were sacrificed, the eyes were enucleated and washed with Ringer's solution, and the corneas were removed and divided into 4 to 8 pieces.

With the plasma, spleen extract, and bits of cornea, cultures were set up. Into a sterile 50 cc. pyrex centrifuge tube 2 or 3 cc. of plasma were introduced, to

which a few drops of tissue extract were added to induce clotting. On top of each clot was placed 1 piece of normal or infected cornea which was then covered with another thin clot of plasma (1 cc.) and spleen extract (Text-fig. 2). The tubes, sealed with sterile corks, were placed in an incubator at 37°C. for 24, 48, and 72 hours.

After incubation the clots, containing their bits of cornea, were removed from the tubes, fixed in Zenker's fluid, embedded in paraffin, sectioned, and stained according to Giemsa's method or with eosin and methylene blue. Phloxine at times was substituted for eosin. Then many sections from each block of tissue were examined for the presence of characteristic inclusions. As controls for the

TEXT-FIG. 2. Appearance of cultures prepared in 50 cc. pyrex centrifuge tubes.

presence of active virus in the cultures, preparations similar to those studied histologically were emulsified and tested in the skin of rabbits.

RESULTS

Numerous observations have been made on the virus of vaccinia, and 2 sets of experiments have been performed with herpetic virus. Inasmuch as corneal tissue from adult rabbits was used, one might suppose that contaminating bacteria would have interfered with the work. Many of the tubes, however, showed no evidence of bacterial contaminations; others contained only a few colonies of organisms that did not disturb the activity of the viruses; and a few were

sufficiently infected with bacteria to be valueless. To circumvent the invalidation of results by bacterial contaminants, experiments were always set up in duplicate and occasionally in triplicate.

In this work Levaditi's strain of neurovaccine produced satisfactory lesions more consistently than did Noguchi's testicular virus. Although on one occasion positive results were obtained with a glycerolated virus, it seemed advisable to use a freshly prepared virus emulsion for each experiment. Corneas infected *in vivo* and *in vitro* showed excellent lesions regularly. The latter, however, were less frequently contaminated.

In each experiment, bits of cornea were fixed for examination after 24 and 48 hours of incubation. Characteristic lesions with inclusion bodies were found in infected tissues after both periods of incubation. If the virus were very potent, however, and if the inoculation were highly successful, 24 hours post-inoculation was found to be a suitable time for examination of the tissues, otherwise 48 hours proved to be more favorable. Some preparations were allowed to remain at 37°C. for 3 days. In these the infected epithelium was extensively disintegrated and unsuitable for histological examination.

Control tissues always revealed definite evidence of growth of epithelial cells in the form of new epithelium filling in the defects caused by scarification (Fig. 13). At times mitotic figures were observed (Fig. 7). Frequently the tissues appeared to be almost as normal as those treated in a similar manner with the exception of being allowed to remain in the animal. If the inoculations were made *in vitro*, the absence of leucocytes was striking. Significant inclusions were never seen in the controls.

Corneas inoculated with vaccine virus showed either discrete lesions separated by normal tissue or a diffuse infection involving all the epithelium. At the sites of infection the epithelium was thickened (Figs. 3, 6, 11, 14) because of an increase both in the number of cells and in the size of individual cells. Numerous typical Guarnieri bodies (Figs. 8, 9, 11, 12, 14) situated in clear spaces within the cytoplasm of epithelial cells were found. These intracellular changes occurred both in the cells present at the time of inoculation and in the new cells (Fig. 12) growing in to fill the defects produced in the corneal epithelium by scarification. On several occasions cells undergoing

mitosis were observed to contain vaccine bodies (Fig. 9). In addition to the Guarnieri bodies, smaller, irregular, basophilic structures that were considered to be Paschen bodies at times studded the cytoplasm of many cells. The end result of the vaccinal infection as studied under the conditions of these experiments was a complete dissolution of the majority of the involved cells (Fig. 3). By testing the material on the skin of normal rabbits, active vaccine virus was demonstrated in the cultures exhibiting characteristic lesions.

The herpetic virus produced striking changes in the infected corneas. In certain areas (Figs. 4, 15) the increase in the thickness of the epithelium produced hummocks. The intracellular bridges disappeared, the cells were swollen, many amitotic giant cells appeared, and numerous typical acidophilic nuclear inclusions were present (Fig. 15). Structures resembling Guarnieri bodies were never seen.

DISCUSSION

The *in vitro* production of pathological pictures similar to those caused *in vivo* by infectious agents has rarely been accomplished. Maximow (9, 10) and Lang (11), however, have reported that certain tissues cultivated and infected *in vitro* with tubercle bacilli evince pathological changes resembling those observed in infected hosts. In spite of the fact that the viruses of vaccinia and herpes simplex can be cultivated in tissue cultures, no one has been able previously to show definitely that characteristic cytologic lesions of these diseases occur in such cultures. Therefore, the method described above is the first one evolved by which typical vaccinal and herpetic lesions may be regularly produced in tissues inoculated and cultivated *in vitro*. Rabbit corneas handled in a special manner were used for this purpose. In view of Andrewe's (2) work, it seems that other viruses and other tissues may be employed for similar studies. The paper that immediately follows indicates that such methods offer a new means of studying and analyzing virus infections and virus immunity.

SUMMARY

Characteristic vaccinal and herpetic lesions, including Guarnieri bodies and acidophilic nuclear inclusions respectively, regularly occur in rabbit corneas infected and cultivated *in vitro* according to the method here described.

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EXPLANATION OF PLATES

PLATE 29

FIG. 1. Section of a cornea immediately after *in vitro* scarification and inoculation. Note the breaks in the epithelial cells induced by scarification. Giemsa. × 155.

FIG. 2. Section of a bit of normal cornea after cultivation for 48 hours in normal plasma and spleen extract. Eosin and methylene blue. × 155.

FIG. 3. Section of a 48-hour vaccinal lesion in a cornea infected and cultivated *in vitro*. Note thickening of the layer of epithelial cells which are beginning to disintegrate. Compare with Figs. 1 and 2. Eosin and methylene blue. × 155.

FIG. 4. Section of a 48-hour herpetic lesion in a cornea infected and cultivated *in vitro*. Giemsa. × 155.

FIG. 5. Corneal epithelial cells 2 hours after inoculation with vaccine virus. Compare with Fig. 6. Eosin and methylene blue. × 380.

FIG. 6. Corneal epithelial cells 48 hours after *in vitro* inoculation with vaccine virus and cultivation in normal plasma. Compare with Fig. 5. Eosin and methylene blue. × 380.

Fig. 7. Mitotic figure in a 48-hour corneal culture. Giemsa. $\times$ 1,000.

Fig. 8. Section of a 48-hour corneal culture infected with vaccine virus. Note Guarnieri bodies and mitotic figure. Giemsa. $\times$ 1,000.

Fig. 9. Section of a 48-hour corneal culture infected with vaccine virus. Note Guarnieri bodies, cell with mitotic figure and Guarnieri body, and beginning dissolution of the epithelial cells. Giemsa. $\times$ 1,000.

PLATE 30

Fig. 10. Section of a cornea infected (2 hours) with vaccine virus *in vivo.* Note the defect caused by scarification. A bit of the cornea was fixed as a control immediately after removal from the rabbit. Compare with Figs. 11 and 12. Giemsa. $\times$ 380.

Fig. 11. Section of a piece of cornea similar to that shown in Fig. 10, with the exception that it was cultivated *in vitro* for 48 hours. Note the difference in thickness exhibited by the layers of epithelium in the two figures. Insert ($\times$ 1,000) shows a Guarnieri body in one epithelial cell engulfed by another. Giemsa. $\times$ 380.

Fig. 12. Section of a piece of cornea similar to that shown in Fig. 10, with the exception that it was cultivated *in vitro* for 48 hours. Note that the defect in the layer of epithelial cells has been filled in with new cells, many of which contain Guarnieri bodies. Insert ($\times$ 1,000) shows a Guarnieri body in one cell engulfed by another. Giemsa. $\times$ 700.
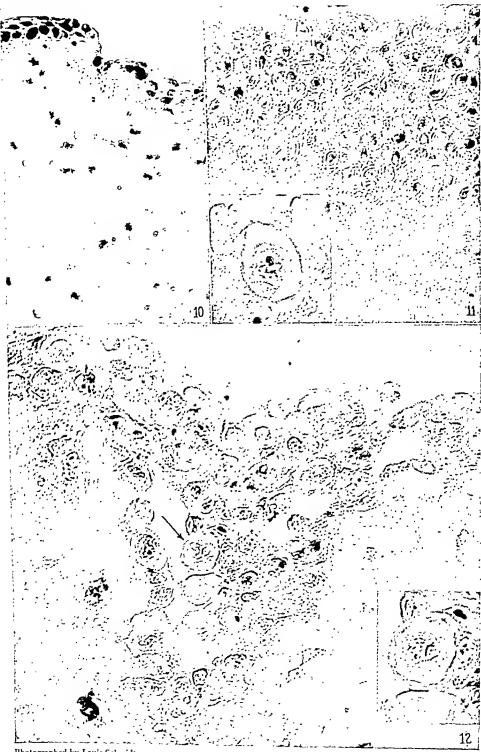
PLATE 31

Fig. 13. Section of a bit of normal cornea cultivated for 48 hours in normal plasma and spleen extract. Note growth of cells filling in defects caused by scarification. Eosin and methylene blue. $\times$ 225 and $\times$ 1200.

Fig. 14. Section of a 48-hour culture of a vaccine virus infected cornea. Note the thickening of the layer of epithelial cells, many of which contain vaccine bodies. Giemsa. $\times$ 225 and $\times$ 1200.

Fig. 15. Section of a 48-hour culture of a herpes virus infected cornea. Note hummock formation, amitotic giant cells, and acidophilic nuclear inclusions. Giemsa. $\times$ 225 and $\times$ 1200.

(Rivers *et al.*: Intracellular changes in tissue cultures)
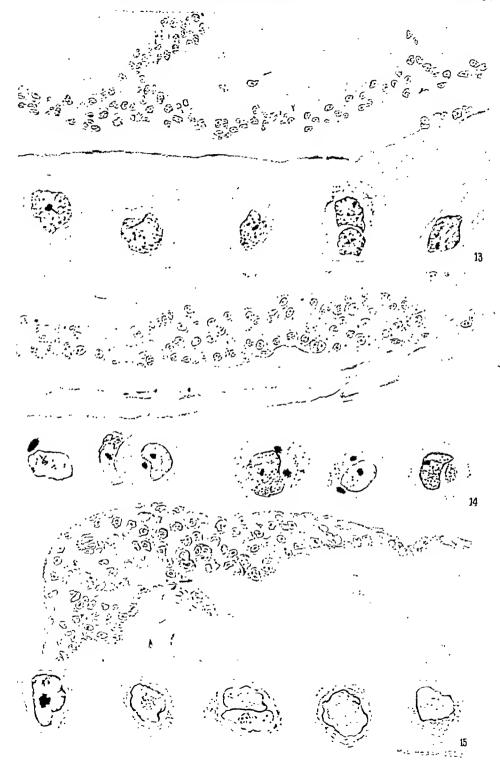
(Rivers *et al.*: Intracellular changes in tissue cultures)

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(Rivers *et al.*: Intracellular changes in tissue cultures)

A STUDY OF VACCINAL IMMUNITY IN TISSUE CULTURES

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In the preceding paper (1) a method of obtaining in tissue cultures visible evidences of infection with vaccine virus was described. The method is applicable to the study of certain phenomena related to vaccinal immunity, and the present communication deals with information secured in this manner.

For the investigation of various immunological problems, tissue cultures or tissue culture methods have been employed by Carrel and Ingebrigtsen (2), Przygode (3, 4), Foot (5), Hadda and Rosenthal (6), Lambert and Hanes (7), Bloom (8), Shwartzman (9, 10), Meyer and Loewenthal (11), Loewenthal and Micseh (12), and others. Such methods have also been used in experimental work on virus immunity. Steinhardt and Lambert (13) attempted to grow vaccine virus in cultures of normal corneal epithelium embedded in antivaccinal plasma. When these cultures were emulsified and inoculated on the skin of a rabbit, no virus was demonstrable. Later, Harde (Steinhardt) (14) reported the results of additional work. She found that cultures of normal or immune corneas in antivaccinal plasma did not support the growth or survival of vaccine virus, while preparations of immune corneas in normal plasma were less "lytic" for the virus. Recently, Nye and Parker (15) attempted to cultivate vaccine virus in normal tissues embedded in normal plasma to which serum from a hyperimmunized calf had been added. In these cultures they were unable to demonstrate virus, while in their control preparations virus was shown to be present.

Fischer's (16) results with the virus of Rous' sarcoma are at variance with those reported above by workers dealing with vaccine virus. He found that serum from immune fowls neutralized active tumor filtrates, but had no deleterious effects on sarcoma cells in tissue cultures, for, when cells from such cultures were injected into susceptible fowls, typical tumors developed. In view of these findings, he concluded that the antibodies in the immune sera were unable to injure virus bound to or situated within cells.

Todd (17) and Andrewes (18) have shown that certain antiviral sera, which inhibit the activity of viruses, are not necessarily "virucidal." From inactive mixtures or from neutral mixtures of immune serum and virus they have been able, under proper conditions, to recover active fowl plague virus and vaccine virus.

Furthermore, Andrewes (19) has reported that, if vaccine virus was injected into the skin 5 minutes before an overneutralizing dose of antivaccinal serum was administered in and around the site of inoculation, the activity of the infectious agent was not completely suppressed; whereas, if the serum was injected first, or if a mixture of virus and serum was injected, no vaccinal lesions developed. From these facts he is prepared to entertain the idea that the protective antibodies in the serum act by rendering susceptible cells insusceptible rather than by killing the virus.

Rivers and Pearce (20) found that it was possible to recover Virus III and vaccine virus from a transplantable rabbit neoplasm for at least 54 and 64 days respectively after a mixture of tumor cells and viruses had been inoculated intratesticularly. At the time when the viruses were recovered, the hosts had long since become refractory to reinoculation from without, and their sera had been "virucidal" for many weeks. Douglas, Smith, and Price (21) have obtained vaccine virus from the brains of recovered animals 41 days after inoculation. By means of cataphoresis, Olitsky and Long (22) demonstrated vaccine virus in the testicles of rabbits inoculated in the skin many months previously. Cole and Kuttner (23) were able at all times to obtain from immune animals the "salivary-gland virus" of guinea pigs, and it appears that a pig once infected continues to harbor the virus indefinitely in spite of a refractory state to reinfection from without.

The above review of the literature concerning virus immunity is not exhaustive. It is given merely to point out some of the discordant results obtained by means of tissue cultures, and to indicate a few phenomena, observed in animals infected with viruses, that are difficult or impossible of analysis in a living host. The experiments described below, in which the visible evidences of vaccinal infection in tissue cultures, inclusion bodies, that is to say, guided or aided the workers, show how certain problems in the virus field may be attacked.

Methods and Materials

Virus.—Levaditi's neurovaccine virus prepared in the testicles of rabbits was employed in all experiments. It was found advisable to use a freshly prepared virus emulsion to which no preservative had been added.

Tissues.—Rabbit corneas prepared and handled as described in the preceding paper (1) constituted the tissues investigated. Normal corneas were obtained from stock animals. Immune corneas were secured from rabbits that had recovered from severe vaccinal infections in the skin or testicles. Neutralizing properties in the blood and a refractory state in the skin develop rapidly after a dermal infection with vaccine virus. An immune state of the cornea, however, develops very slowly under such conditions, and at times a completely refractory

condition requires several months for its appearance. In this work, no cornea was considered immune until it had been tested *in vivo* with an extremely potent virus and found refractory, or until it had recovered from the vaccinal kerato-conjunctivitis induced by the test inoculation. Furthermore, it is not expedient to test one eye and then use the other, because both eyes do not always simultaneously attain an absolutely refractory state.

Plasma and Serum.—For each experiment fresh normal plasma or serum was collected from stock rabbits. Immune plasma or serum was obtained from rabbits that had recovered, 2 to 4 weeks previously, from severe vaccinal infections. The amount of immune plasma or serum used at any time in the experiments was known to be more than sufficient for the neutralization of the virus employed.

Tissue Extract.—Tissue extract was always secured from the spleens of rabbits that had supplied the normal corneas and normal plasma.

Cultures.—The methods of inoculating the tissues, and of preparing, examining, and testing the cultures were similar to those described in the preceding paper (1).

EXPERIMENTAL

Numerous experiments have been performed, in which various combinations of normal and immune corneas with normal and immune sera, or with normal and immune plasmas, were tested for their reactions to, or effects on vaccine virus. For instance, bits of normal corneal tissue were immersed in immune serum for 2 hours at 37°C., washed in Ringer's solution, placed in an emulsion containing vaccine virus for 2 hours, again washed in Ringer's solution, and finally cultivated in normal plasma and spleen extract. The reverse experiment, in which the bits of corneal tissue were first placed in contact with vaccine virus and then with immune serum, was also made. At times the tissues were allowed to remain in contact with immune serum or vaccine virus overnight in the icebox. The results of such experiments have been irregular, and, inasmuch as no definite conclusions can be derived from them, a report concerning them will not be made at this time.

Certain experiments of other sorts, properly performed and controlled, yielded constant results. They were the ones in which small pieces of normal corneas, inoculated with vaccine virus either *in vivo* or *in vitro*, were cultivated in normal and in antivaccinal plasma for 24 and 48 hours, or in which immune corneas were removed from animals, washed for 2 hours in Ringer's or Tyrode's solution, placed

in a virus emulsion for 2 or 3 hours, and finally cultivated for 24 and 48 hours in normal and in immune plasma. It seems unnecessary to give in detail all the experiments. Some of the typical ones, with their results, will be described however.

Development of Vaccinal Lesions in Bits of Normal Corneas Cultivated in Antivaccinal Plasma

In the study of vaccinal lesions that developed in normal corneas cultivated in antivaccinal plasma, 5 experiments, in which the corneas were inoculated *in vivo*, and 7, in which they were infected *in vitro*, were performed. Of the former, in one of which glycerolated virus was used, all resulted successfully. Of the latter, 5 resulted successfully; in one the infection was so mild that no conclusions could be drawn, and in another the cultures were sufficiently contaminated with bacteria to be rendered valueless. 2 experiments in which the corneas were inoculated *in vivo*, and 1 in which they were infected *in vitro* will be described in detail.

The Development in Normal Corneas Infected in Vivo and Cultivated in Normal and in Immune Plasma

Experiment XIV. May 14, 1929.—Freshly prepared Levaditi neurovaccine was used. Immune plasma, known to possess sufficient neutralizing power, was collected. Both eyes of 2 normal rabbits were thoroughly cocainized, scarified, and inoculated with vaccine virus. 2 hours later normal plasma was obtained from the animals. Then the rabbits were sacrificed, the eyes were enucleated, and finally the spleens were removed for tissue extract. The eyes were washed in Ringer's solution in order to remove as many contaminating bacteria as was possible. The 4 corneas were removed and divided into 20 pieces, 4 of which were immediately placed as controls in Zenker's fluid. The remaining 16 were treated in the following manner:

A.—In clots of normal plasma and spleen extract, 4 pieces of cornea were cultivated at 37°C. for 24 hours, after which 2 were fixed in Zenker's fluid and examined for vaccinal lesions, and 2 were carefully removed from the plasma medium, gently washed in Ringer's solution, ground in a mortar, and injected into the skin of a normal rabbit as a test for the presence of active vaccine virus.

B.—4 pieces were treated in a manner similar to that employed under A with the exception that immune plasma was used instead of normal.

C and D.—Duplicates of A and B respectively, with the exception that the cultures were incubated 48 hours instead of 24.

The results of the above experiment are summarized in Table I and clearly show that normal corneas, inoculated with vaccine virus *in vivo* (2 hours) and then cultivated *in vitro* in antivaccinal plasma, developed typical lesions with Guarnieri bodies in the presence of antibodies in the immune plasma. Furthermore, when bits of normal corneas, inoculated and cultivated in a similar manner, were removed from the immune plasma, washed in Ringer's solution,

TABLE I

Summary of Experiments XIV, XVI, and XVII

Exp.	Cultures	24 hours			48 hours		
		Sections	Rabbit inoculations		Sections	Rabbit inoculations	
			Washed	Unwashed		Washed	Unwashed
XIV	N.C. + N.P.	+++	++++	0	++++	++++	0
	N.C. + I.P.	++	++++	0	+++	+++	0
XVI	N.C. + N.P.	?	0	—	+	0	+++
	N.C. + I.P.	?	—	—	+	++	?
XVII	N.C. + N.P.	++	0	+++	+++	0	+++
	N.C. + I.P.	+++	+	±	++	+	+

N = normal; C = cornea; I = immune; P = plasma; O indicates that test was not made; ? indicates doubtful lesions or doubt regarding presence of active virus; — indicates absence of lesions or absence of active virus; + s indicate the severity of the lesions with Guarnieri bodies as seen in sections of the bits of cornea, or the amount of active virus as determined by the size of the lesions at the sites of inoculation of the bits of cornea in the skin of rabbits.

emulsified, and injected into the skin of a normal rabbit, an abundance of active vaccine virus was demonstrated. The lesions in the bits of cornea cultivated in immune plasma were not as extensive as were those in the pieces of tissue set up in normal plasma. Nevertheless, the lesions were definite and frequently involved the new cells growing in to repair the defects caused by scarification. Inasmuch as these young cells were formed after the bits of cornea were placed in immune plasma,* the indications are that new cells arising from old infected

* This is indicated by the fact that in the controls, promptly fixed in Zenker's fluid after the inoculated eyes were enucleated, no growth of cells had taken place and no Guarnieri bodies were present.

cells or in the immediate vicinity of involved cells are capable of becoming infected even when the tissues are growing in a medium of immune plasma.

Experiment XVI.   June 4, 1929.—The inoculations, collections of normal and immune plasmas, and preparations of cultures, were similar to those described in Exp. XIV.   The following tests and controls, subsequently handled as indicated, were set up.

1 bit of inoculated normal cornea in normal plasma and spleen extract incubated 24 hours; examined histologically.

1 bit of inoculated normal cornea in normal plasma and spleen extract incubated 24 hours: ground up with at least 1 cc. of the plasma and injected into the skin of a rabbit.

2 bits of inoculated normal cornea in immune plasma and spleen extract incubated 2 hours; examined histologically.

2 bits of inoculated normal cornea in immune plasma and spleen extract incubated 24 hours; removed from the plasma clots, washed in Ringer's solution, emulsified, and injected into the skin of a normal rabbit.

2 bits of inoculated normal cornea in immune plasma and spleen extract incubated 24 hours; ground up with at least 1 cc. of the plasma and injected into the skin of a rabbit.

1 tube of immune plasma and spleen extract incubated at 37°C. for 24 hours. Then the plasma was tested for its neutralizing power.

1 tube of immune plasma and spleen extract placed in the icebox for 24 hours. Then the plasma was tested for its neutralizing power.

Duplicates of the above preparations were set up and incubated at 37°C. or stored on ice for 48 hours instead of 24.

The results of Experiment XVI are shown in Table I.   The control portions of immune plasma held in the icebox and in the incubator for 24 and 48 hours were found to be more than capable of neutralizing the amount of virus in the bits of cornea at the beginning of the experiment.   It seemed, however, that both of the 48-hour specimens had lost some of their neutralizing properties.   This phenomenon is undergoing further investigation.   There was very little evidence of vaccine virus either in the sections of the 24-hour cultures or in the rabbits inoculated with emulsions of the cultures.   This was equally true of preparations of normal cornea in normal or in immune plasma. The demonstration of virus in the 48-hour cultures of normal cornea in immune plasma was accomplished through the removal, by gentle washing, of the immune plasma from the bits of tissue before they were

emulsified for test inoculations in rabbits. The failure to separate the infected tissues from the immune plasma may account for the fact that other workers have been unable to demonstrate active virus in cultures of normal tissues infected and then grown in immune plasma. At times,—this fact is well exemplified by the next experiment,—it was possible to demonstrate virus in such cultures even when the pieces of cornea had not been entirely freed from the antivaccinal plasma.

The Development in Normal Corneas Infected in Vitro and Cultivated in Normal and in Immune Plasma

Experiment XVII. June 11, 1929.—This experiment was a repetition of Experiment XVI with 2 exceptions, viz., the corneas were inoculated *in vitro* instead of *in vivo*, and 2 pieces of the tissue were chosen for controls after they had been inoculated with vaccine virus and before the cultures were set up. 1 of the control bits of cornea was ground up with 0.5 cc. of normal plasma, the other with 0.5 cc. of immune plasma. These emulsions, tested in the skin of a normal rabbit, showed that the immune plasma neutralized the virus in the freshly inoculated cornea while the normal plasma did not. Thus it was demonstrated that 3 cc. of immune plasma, the amount used in the cultures, was at least 6 times greater than the amount necessary to neutralize the virus in the bits of cornea at the time when the cultures were set up.

From the results of Experiment XVII, which are summarized in Table I, it is evident that normal corneas infected *in vitro* and cultivated in antivaccinal plasma developed typical vaccinal lesions with Guarnieri bodies and that such cultures contained active vaccine virus. There was little or no difference in the size of the lesions produced by the washed and unwashed bits of cornea from the 48-hour cultures. A difference was apparent, however, in the tests made with the tissue from the 24-hour cultures.

Reaction of Immune Corneas to Vaccine Virus

In the investigations concerning the nature of the *in vitro* reaction of immune corneas to vaccine virus, 6 experiments were performed. 2 of these must be disregarded, inasmuch as the corneas were not tested *in vivo* for immunity before being used in the cultures. For immunity to vaccine virus is relative, and certain tissues more slowly

attain a completely refractory state than do others. This is particularly true of corneal epithelium. In fact, one cornea of an animal may be refractory while the other is slightly susceptible. Moreover, the results of skin tests are not reliable indicators of the state of corneal immunity. Therefore, in order to be correctly informed concerning the state of immunity of any cornea, one must test it directly with a freshly prepared potent virus. In 3 of the 4 experiments which were properly controlled, the immune corneas exhibited no typical vaccinal lesions, while in 1 the corneas showed evidences of a very mild infection associated with a few Guarnieri bodies. 2 of the experiments will be described in detail.

Immune Corneas Infected in Vitro and Cultivated in Normal and in Immune Plasma

Experiment XI. Jan. 18, 1929.—A normal rabbit received multiple intradermal inoculations of vaccine virus. Feb. 8, 1929, the animal was reinoculated in the skin and corneas. A mild reaction occurred in the skin, and a moderately severe one in the eyes. April 16, 1929, the rabbit was sacrificed for immune plasma and immune corneas. A normal animal was likewise sacrificed for plasma, corneas, and spleen extract. A fresh testicular virus emulsion was prepared. The immune and normal corneas were inoculated with vaccine virus *in vitro* (3 hours) in the usual manner; cultures were prepared and incubated at 37°C. for 48 hours; the tissues were than fixed and examined with the following results.

1.—Normal corneas infected and cultivated in normal plasma revealed severe vaccinal lesions with numerous Guarnieri bodies.

2.—Immune corneas infected and cultivated in normal plasma evidenced a very mild infection associated with a moderate number of vaccine bodies.

3.—Immune corneas infected and cultivated in immune plasma showed an exceedingly mild infection with only an occasional Guarnieri body.

In the above experiment, although evidences of a mild infection were found in the immune corneas, a definite difference in the extent of the involvement of the normal and immune tissues was evident. The experiment was repeated with an animal that had been immunized and tested in a manner similar to that used upon the immune animal above, and no evidences of a vaccinal infection were found in the immune corneas, while moderately severe lesions were noted in the normal corneas. In these experiments, no tests for the presence of

active vaccine virus in the cultures were made. Such tests, however were conducted on 2 other occasions, and the results, one set of which will be described below, were identical in both instances.

Experiment XVIII. Three rabbits received multiple intradermal inoculations of vaccine virus, Jan. 24, Feb. 4, and Feb. 18, 1929, respectively. June 3, 1929, the animals were reinoculated in the skin and corneas. All were refractory to reinfection of the skin. The first animal, however, had a mild "take" in the right eye, none in the left; the second evidenced a mild infection in both eyes; the third showed a reaction in the right eye, none in the left. The lesions in the eyes rapidly healed, and the corneas were used as immune tissues. The corneas that had evidenced "takes" and had recovered were labeled A, while those that had shown no reaction were marked B. A s and B s were run in parallel series, but, inasmuch as the results in the 2 series were identical, they will not be described separately.

June 18, 1929, the 3 immune rabbits were sacrificed, and the corneas were removed, scarified, divided into 4 pieces each, and washed for 2 hours in Tyrode's solution at 37°C. Then, the bits of cornea were immersed for 2 hours in a fresh virus emulsion at 37°C. Immune plasma was collected from rabbits recently (2 to 4 weeks) recovered from severe vaccinal infections in the skin. Normal plasma, normal corneas, and spleen extract were obtained from stock rabbits. The normal corneas were scarified and infected in a manner similar to that employed for the immune tissues. Either 4 or 8 tubes of each combination of the materials enumerated above were prepared. One half of the preparations was incubated 24 hours, while the remaining half was held at 37°C. for 48 hours. At the end of each period of incubation the preparations were again divided; the bits of cornea in one half were fixed and stained for histological examination, while those in the other half were carefully removed from their plasma clots, washed in Ringer's solution, emulsified, and injected into the skin of normal rabbits. Lesions produced by these intradermal inoculations were excised, emulsified, and rubbed on the scarified skin of another set of normal rabbits in order to demonstrate typical vaccinal pustules. Control specimens of immune plasma held on ice or in the incubator for 24 and 48 hours were tested for neutralizing properties. The results of the experiment are given in detail below.

24-hour Preparations

1.—Normal cornea in normal plasma: Mild vaccinal lesions found, virus present.

2.—Immune cornea in normal plasma: No vaccinal lesions observed, virus present.

3.—Immune cornea in immune plasma: No vaccinal lesions observed. Virus not demonstrable.

4.—Specimens of immune plasma held on ice and in the incubator appeared to have retained full neutralizing power.

48-hour Preparations

5.—Normal cornea in normal plasma: Pronounced vaccinal lesions observed, large amount of virus present.

6.—Immune cornea in normal plasma: No vaccinal lesions found, virus present but less in amount than that in tissue of 5.

7.—Immune cornea in immune plasma: No vaccinal lesions seen, virus present but less in amount than that found in the cultures of 6.

8.—Specimens of immune plasma held on ice and in the incubator seemed to have lost some of their neutralizing power. They were still capable, however, of neutralizing an amount of virus greater than that taken up by the bits of cornea during the process of inoculation.

From the results of Experiment XVIII it appears that the epithelium of normal and immune corneas did not react similarly to vaccine virus, as evidenced by the fact that typical vaccinal lesions with Guarnieri bodies were observed only in the former. Virus, however, was demonstrated in the preparations of normal corneas in normal plasma, in the 24-hour cultures of immune corneas in normal plasma, and in the 48-hour cultures of immune corneas in normal and in immune plasma. Furthermore, the amount of virus in the 48-hour preparations of immune corneas seemed, as determined by the size of lesions produced in rabbits, to be greater than that in the 24-hour cultures. At present, it is impossible to state whether this apparent increase in the amount of virus was due to an actual multiplication of the infectious agent. Nevertheless, the striking fact remains that vaccine virus can at least survive for 48 hours in cultures of immune corneas in normal plasma and to a less extent in preparations of immune corneas in antivaccinal plasma.

DISCUSSION

It should be noted that the *in vitro* vaccinal infection of normal corneal epithelial cells and the *in vitro* refractory state exhibited by immune corneal epithelium occurred without the direct intervention of such structures as cells of the reticulo-endothelial system and white blood cells. What part, if any, the various tissues of the body played in bringing about the refractory state of the immune corneas before they were removed from the body is not known.

Adequate explanations of some of the observations reported in this

paper must await further experimental work. Interpretations for a few of the results, however, are at hand, but even these are associated with a certain amount of speculation. The fact that vaccinal lesions with Guarnieri bodies developed in normal corneas inoculated *in vitro* and then cultivated in antivaccinal plasma does not appear unusual, provided one assumes that the activity of virus situated intracellularly could not be influenced by the antibodies in the immune plasma. This assumption at least agrees with the experimental observations of Rous and Jones (24) who found that intracellularly situated typhoid bacilli and red blood cells were not susceptible to such injurious agents as potassium cyanide and antisera. The results of the experiments in which immune corneas* were cultivated in normal and in immune plasma appear to support the idea of Todd (17) and Andrewes (18) that substances protective against viruses are not necessarily virucidal and also to fall in line with the observations recorded concerning the persistence of viruses in immune animals (20, 21, 22, 23).

SUMMARY

Normal corneas inoculated *in vitro* with vaccine virus and then cultivated in antivaccinal plasma developed typical vaccinal lesions associated with Guarnieri bodies. In such cultures, after an incubation period of 24 or 48 hours, active vaccine virus was demonstrated by means of appropriate methods.

Immune corneas inoculated *in vitro* with vaccine virus and then cultivated in normal or in antivaccinal plasma revealed either very mild vaccinal lesions or none at all. In some of the cultures after 24 and 48 hours of incubation, active vaccine virus was demonstrated.

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* Results reported in this paper are different in certain respects from those secured by Dr. Andrewes (personal communication to Dr. Rivers). Such differences, no doubt, may be accounted for by the fact that he conducted his experiments with Virus III in testicular tissue cultures made according to Maitland's technique.

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CUTANEOUS REACTIONS TO THE POLYSACCHARIDES AND PROTEINS OF PNEUMOCOCCUS IN LOBAR PNEUMONIA

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PLATE 32

Clough, 1915, (1) employing a solution of alcohol precipitable protein derived from pneumococci, tested patients suffering from pneumonia by intradermal injections. There resulted in some instances a slightly elevated discrete papule surrounded by an indefinite zone of hyperemia; all of the reactions faded in 24 hours. Little or no difference was noted between the reactivity of patients with pneumonia and normal individuals, nor was the reaction affected by the stage of the disease in which the test was made. Weil, 1916, (2) prepared material from cultures of Type I pneumococcus. He allowed the organisms to autolyze at 37°C. for 2 hours, followed by heating at 60°C. for 1 hour. 0.1 cc. to 0.2 cc. of the supernatant fluid of this solution was used for skin tests. He obtained no reaction in patients tested during the acute phase of the disease. However, 1 to 21 days after crisis, injections produced a circumscribed erythema with slight infiltration at the point of puncture. A few cases did not react at any time; normal persons and patients suffering from other diseases gave no uniform response. In 1917, Steinfeld and Kolmer (3) carried out a series of skin tests, employing heat killed Type I, II, and III pneumococci. The reactions were read 48 hours after the injection; when positive the local lesion lasted 4 to 5 days. Six of 19 pneumonia patients gave positive reactions with one or another of the test organisms. No relation was noted between the type of pneumococcus eliciting the skin response and the causative organism of the disease. All of the positive reactions were obtained in patients tested after the crisis. Tests on control individuals were recorded as being negative. Weiss and Kolmer, 1918, (4) employed in a study of the cutaneous reaction of pneumonia patients, so-called pneumotoxin prepared by dissolving washed pneumococci in 2 per cent sodium choleate. This material was freshly made and standardized in terms of minimal lethal doses for guinea pigs. The reactions were read 48 hours after the injection. In adults positive reactions were obtained in 100 per cent of 31 acute cases. The period of reactivity ranged from the fifth day (2 days before crisis) to the thirteenth day (6 days after crisis) of disease. In children, tests were positive during the acute phase of illness and negative after recovery. No correlation was noted between a

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positive test and the type of pneumococcus derived from the sputum. Bigelow, 1922, (5) prepared several varieties of test materials; they consisted of heat killed pneumococci, autolysates, and solutions. In some instances the material was derived from pooled cultures of Types I, II, III, and Group IV pneumococci; other preparations were prepared from organisms of a single type. In tests on 104 patients, he described two types of reaction. One of these was designated as . type specific. It was described as an indurated papule surrounded by an areola, which reached its height 28 to 32 hours after inoculation. This form of response was best obtained with autolysates. It was considered by the author to be type specific since in 8 of 11 cases the material eliciting a positive reaction corresponded in type to that of the pneumococcus recovered from the patient. In 3 patients this correlation did not occur. The second or "common" reaction was obtained with most of the preparations. Lesions of this character reached their height in 18 hours and faded in 24 to 36 hours. It was obtained in 42.3 per cent of those tested and, in most instances, was elicited after crisis had taken place. Forty-five per cent of control individuals also gave this reaction. The author concludes that positive tests occur most commonly at the time during which antibodies would be present in greatest concentration. Herrold and Traut, 1927, (6) employed filtrates derived from a 5 day culture of an avirulent Type I pneumococcus. Reactions, when positive, appeared in about 12 hours and reached the maximum after 18 hours. Readings were made at the end of 24 hours. Of 38 patients acutely ill with pneumonia, 73 per cent failed to react, while only 15 per cent of normal persons, gave negative tests. Eight patients were repeatedly tested; one of these, negative at first, later became positive; another, originally positive, subsequently became negative; 2 were positive and 4 negative throughout the period of observation. They state that they found no relation between the material used for positive tests and the type of pneumococcus causing the infection.

From this review of the literature it will be seen that uniform results have not been obtained in pneumonia patients tested intracutaneously with pneumococcus products. It will be further noted that, with the exception of Clough (1) who used pneumococcus protein, the tests have been made with autolysates, heat killed organisms, solutions of pneumococci, and culture filtrates. Materials of this character are known to contain a variety of bacterial products, such as protein, carbohydrate, pneumotoxin, hemolysin, and the purpura producing substance, as well as other derivatives of enzyme action, in varying concentrations. Attention is directed to this point since the results reported in this paper demonstrate that the composition of the material used for injection influences the character of the reaction.

In the experiments presented in the present communication measured quantities of two distinct constituents of the pneumococcus cell

were separately employed for intradermal injection into patients acutely ill with and convalescent from lobar pneumonia. The material used for testing consisted of: 1. The purified, protein-free, carbohydrates of Types I, II, and III pneumococci,—the so-called soluble specific substances; 2. The somatic proteins of Pneumococcus. This material, the acetic acid, precipitable fraction, consists largely of nucleo-protein. A description of both carbohydrate and protein is given further on. It has been shown by Avery and Heidelberger (7) that the pneumococcus carbohydrates are the type specific components; they react only in homologous type specific anti-sera, and, in purified form, are not antigenic. The protein fraction, on the other hand, is a common constituent of the pneumococcus species; antibodies elicited by immunization with it, react with protein derived from any strain of this organism.

In addition to the intradermal injection of the polysaccharides and protein, the experiments were supplemented by obtaining serum from the patients just prior to the skin test, and titrating for the presence or absence of antibodies reactive with the test solutions.

Material and Methods

Skin Testing Materials. 1. Soluble Specific Substances.—Type specific polysaccharides from each of three pneumococcus Types (I, II, and III) were employed. They were obtained in purified state according to the method employed in this laboratory by Heidelberger and Avery (8). As demonstrated by Heidelberger, Goebel, and Avery (9), these substances possess the chemical properties of complex sugars; they contain no phosphorous, no sulfur, and give none of the usual protein color tests. Type II and Type III substances are nitrogen-free. The Type I substance differs from the other two in containing nitrogen as an apparently essential component. The total nitrogen present in Type I is 5 per cent, half of which is present in the amino form. Despite the presence of nitrogen, the substance fails to give any of the protein color reactions. For skin tests, the polysaccharides were dissolved in *freshly* prepared physiological salt solution in such concentration that 0.1 cc. of solution contained 0.01 mgm. of specific polysaccharide. For purposes of sterility the solutions were heated at 100°C. for 10 minutes before being used.

2. Pneumococcus Protein.—The material was prepared according to the method described by Avery and Morgan (10). Cultures of an R strain of pneumococcus originally derived from Type II S organisms served as the source from which the protein was obtained. The preparations were filtered through Berkefeld filters and tested for sterility before being used for injection. The material was stand-

ardized by nitrogen determinations and diluted in fresh physiological salt solution so that 0.1 cc. of solution contained 0.01 mgm. of protein.

In each instance the material was injected in 0.1 cc. amounts into the skin on the flexor surface of the forearm.

Titration of Sera for Antibodies.—Blood, obtained from patients by venapuncture, was allowed to clot, centrifuged, and the clear serum pipetted off. Tests for precipitins reactive with carbohydrate and protein were separately carried out. 0.2 cc. serum diluted with 0.3 cc. physiological salt solution was mixed with 0.5 cc. of varying dilutions of the precipitinogens. Readings were made after the tubes had been allowed to incubate at 37°C. for 2 hours and then placed in the icebox over night. It was found that type specific antibodies were more easily demonstrable by agglutination tests with the intact type specific cells. Consequently this test was more commonly used. 0.5 cc. of varying dilutions of serum was mixed with 0.5 cc. of heat killed organisms of each of Types I, II and III. The tubes were incubated at 37°C. for 2 hours and kept on ice over night. A positive reaction was detected by the presence of the characteristic disc formation.

On admission to the hospital patients were injected intradermally with Types I, II and III carbohydrate solutions, protein solution, and a control injection of fresh physiological salt solution. The tests were often repeated several times both during the acute phase of the illness and after crisis. Freshly prepared materials were always employed for injection. This precaution was considered to be of considerable importance since it was found that solutions which had been standing for several days often gave rise to immediate reactions which were deceptive. In addition to skin tests, serum obtained, in most instances at the time of intradermal injection, was titred for antibodies reactive with the carbohydrates and protein. Typing of the pneumococcus derived from the sputum of the tested patients was also carried out.

Cutaneous Reactions to Specific Polysaccharides

Observation on the skin reaction of nineteen individuals suffering from lobar pneumonia have been made following injection of 0.01 mgm. of each of the type specific polysaccharides. Classified on the basis of the pneumococcus type derived from the patient, the cases may be divided into: 11 cases of Type I infection; 3 of Type II; 2 of Atypical Type II; and 3 of Group IV. Unfortunately, no instances of Type III pneumococcus infection were available during the period of this investigation.*

* While this communication was in press a patient, convalescent from Type III pneumococcus pneumonia, has shown a positive reaction with the Type III polysaccharide. The patient's serum contained type-specific antibodies at the time of the positive test.

In the 11 cases of Type I infection, 10 reacted to Type I S carbohydrate and none to the Type II or III substances. Of the 3 Type II cases one reacted to Type II polysaccharide, whereas all three were negative to the sugars derived from Types I and III. The 2 atypical Type II and the 3 Group IV cases were entirely negative.

With regard to the time of appearance of a positive reaction, none of the cases before recovery reacted positively. Of the 11 cases in which a positive test was obtained, 10 reacted at the time of recovery. One patient, suffering from Type I pneumococcus pneumonia did not give a positive skin test until the twenty-sixth day after recovery from the acute phase of the illness. He had persistent fever, and evidence of pleurisy during this time and only after these signs of persistent infection began to subside did the skin test become positive.

A positive reaction, when obtained, was very striking and may be described as follows:

Ten to twenty minutes after the intradermal inoculation, there appears at the site of injection a wheal-like swelling with intense white edema. Surrounding the wheal, a zone of erythema appears which becomes increasingly larger and more intense. The edges are irregular due to the "pseudopods" of erythema extending in different directions. The height of the reaction occurs between 30 and 60 minutes after the inoculation. (See Figs. 1 and 2.) Within another hour the acute phase has entirely faded; a gradual regression then takes place leaving a firm, pale, edematous area which may require 24 hours or longer to disappear completely. Reactions of this character have been found to be strictly type specific and to occur only with the polysaccharide corresponding to the serological type of the infecting organism. At the site of injection of the carbohydrates of heterologous types and of the salt solution, no reaction appeared. The "wheal and erythema" form of reaction, just described, differs strikingly from the delayed response elicited by the bacterial protein.

In testing the sera of patients, it was found that in all instances in which a positive skin reaction was elicited with the specific carbohydrates, agglutinins for the homologous type of pneumococcus and precipitins for the reacting polysaccharide were present. Furthermore, the time at which specific antibodies were demonstrable in the circulation has been coincident with the development of a positive skin reaction. However, the fact that individuals may possess circulating specific antibodies without responding to the intradermal injection of the homologous specific substance will be subsequently shown.

Ten of the 11 cases in which a definite skin response to the homolo-

gous specific polysaccharide was obtained, suffered from Type I pneumococcus infection. Eight of these cases were treated with Type I antipneumococcus horse serum in 100 cc. doses at six hour intervals until recovery. Regardless of the amount of serum administered the skin reaction did not become positive until the patient had clinically recovered. The investigations are not as yet sufficiently extensive to determine the exact relations between specific antibodies introduced therapeutically and the occurrence of a positive skin reaction. However, the results are suggestive that the production of a local response with the specific polysaccharide may, under certain conditions, be helpful in determining when sufficient serum treatment has been given. Studies are being continued in an attempt to determine this point.

The single instance of Type I infection, which failed to react to the Type I carbohydrate occurred in a patient who developed pneumococcus meningitis and died on the twentieth day of disease. This patient, after receiving 800 cc. of serum, possessed demonstrable circulating Type I antibodies, but gave only doubtful or negative skin test. Another individual, after recovering from the acute phase of the illness, continued to run a low grade fever and showed signs of pleurisy. He reacted positively to the Type I specific substance only after beginning cessation of infection. These two cases suggest that persistence of infection, even though specific antibodies are present, may inhibit the skin response.

In two patients suffering from Type I pneumococcus pneumonia, no serum was administered. In both instances a positive skin response was obtained after recovery, indicating that a positive test is not dependent upon the presence of therapeutic serum. Of three Type II cases included in this series one gave a typical positive reaction to the Type II specific polysaccharide. The reaction first appeared as the result of a test made the day after crisis; the patient's serum at this time contained Type II agglutinins and precipitins. The other two Type II patients failed to react even to 0.1 mgm. of specific substance. These failures occurred despite the fact that the blood serum, in each case, contained specific Type II antibodies and that all evidence of infection had completely subsided. As formerly noted the two atypical Type II and 3 Group IV cases of pneumococcus in-

fection gave no response at any time to the skin tests with the polysaccharides of Types I, II, or III.

In all patients, tests were repeated every few days during convalescence. Those patients in whom the reactions were positive on several occasions and subsequently became negative, ceased to react at the time of disappearance of specific antibodies from the circulation.

In Table I a protocol of five cases is given, which serves to show the time of appearance and duration of skin sensitivity in relation to both recovery of patient and occurrence of demonstrable specific antibodies in the circulation.

Cases Ka. and La. suffered from Type I pneumococcus infection and were treated with Type I antipneumococcus horse serum. Case Ka. is interesting in that skin sensitivity to Type I carbohydrate and circulating Type I antibodies have persisted for 127 days after recovery; observations on this patient are being continued. Case La. first reacted on the day after recovery; three days later his capacity to react had ceased coincident with the disappearance of specific agglutinins and precipitins. Patient Ca., the case of pneumococcus meningitis referred to above failed to give a definite positive reaction at any time. Cases St. and Mo. were instances of spontaneous recovery from Type I and Type II pneumococcus infections, respectively. They each gave typical responses after crisis and continued to do so, as long as demonstrable circulating antibodies were present. In the other patients, who gave a positive skin test, the same relations were found to hold as those presented in Table I.

The local reaction resulting from the intradermal injection of 0.01 mgm. of the type specific polysaccharides presented three distinctive characteristics; first, the carbohydrate eliciting a response was always homologous in type to that of the infecting organism; second, both the gross appearance of the lesion and the rapidity with which it developed and retrogressed were characteristic of the typical wheal and erythema type of reaction; third, the capacity to react, when evident, developed at the time of the patient's recovery, and was, in this group of cases, correlated with the presence of homologous type specific agglutinins and precipitins.

Cutaneous Reactions to Pneumococcus Protein

The local reaction resulting from the intradermal injection of 0.1 mgm. pneumococcus protein presented distinct differences from that

Skin Reactions with Pneumococcus Polysaccharides in Rela...

Day of disease		1	2	3	4	5	6	7	8	9	10	11	12
Ka. Age 14	Agglutinins						—	+++					++
	Precipitins						—	+					+
Type I	Skin Test.						neg.	pos. C					pos.
La. Age 34	Agglutinins						—	+			—		
	Precipitins						—	+			—		
Type I	Skin Test.						neg.	pos. C	pos.		neg.		
Ca. Age 55	Agglutinins				—	++		+++					+++
	Precipitins				—	+		+					+
Type I	Skin Test.				neg.	neg.	neg.	neg.					?
St. Age 33	Agglutinins			—									
	Precipitins			—									
Type I	Skin Test.			neg.				C				pos.	
Mo. Age 33	Agglutinins				—			++				+++	
	Precipitins				—			+				+	
Type II	Skin Test.						neg.	pos. C				pos.	

Agglutinin titre in terms of highest positive serum dilution. Agglutination characterized by disc formation.

    — = Completely negative.

    ± = Doubtful agglutination in serum dilution of 1:2.

    + = Agglutination in serum dilution of 1:2.

    ++ = Agglutination in serum dilution of 1:10.

    +++ = Agglutination in serum dilution of 1:20.

    ++++ = Agglutination in serum dilution of 1:40.

E I

Titre of Circulating Specific Antibodies and Recovery of Patient

14	15	16	17	18	19	20	21	22	23	24	26	27	28	29	49	97	109	133
								++ +					+ +		+ +	+ +	+ +	+ +
								pos.					pos.		pos.	pos.	pos.	pos.
									− −									
									neg.									
			?			Death Meningitis												
+ +									+ ±					± −			
pos.									pos.					?			
-+++ +					++++ +									(47) −			
pos.					pos.									neg.			

Precipitin test = 0.3 cc. serum + 0.2 cc. salt solution + 0.5 cc. 1–40,000 dilution of polysaccharide.

Skin test:

pos. = positive reaction.

？ = doubtful reaction.

neg. = negative reaction.

C = day of crisis.

TABLE II

Skin Reactions to Pneumococcus Proteins in Relation to Titre of Circulating Protein Precipitins and Recovery of Patient

Type I

Day of disease	Ka. age 15						Ga. age 17				*Co. age 26			
	6	7	12	22	29	36	3	4	6	13	4	16	23	36
Protein Precipitin Titre. 1-500	++		+	+++	+++	+++	+++	+++		+++	++	++	++	++
1-1000	++		±	+++	+++	+++	++	+++		+++	±±	++	+++	++
1-2000	+	Crisis.	+	±	+	+	±	+++	Crisis.	+++	+	+	+	+
1-4000	±		-	±	±	±	+	+++		++	±±	-	-	±±
1-8000	-		-	-	-	-	+	+++		+	±±	-	-	-
1-16000	-		-		-		±	+		±	-	-		-
Skin test.	neg.		?	pos.	pos.	pos.	neg.	neg.		pos.	neg.	neg.	neg.	neg.

* Patient Co. had a prolonged illness followed by recovery.

Type II

Day of disease	Fl. age 33					Lo. age 24				
	4	8	10	20	27	2	5	*7	17	24
Protein Precipitin Titre. 1-500	0		+	±±	++	++	++	++	+++	+++
1-1000	0		±	++	++	+++	+++	+++	+++	+++
1-2000	0	Crisis.	±	±	±±	++	+++	±±	+++	+++
1-4000	0		±	-	-	±	++	+	++	++
1-8000	0		-	-	-	±	+	±±	±	±±
1-16000	0		-					-		
Skin test.	neg.		neg.	pos.	pos.	neg.	0	?	pos.	pos.

* Day of crisis.

	Type Atypical II								Group IV	
	Wa. age 21				Ne. age 42				IIa. age 36	
Day of disease	1	8	17	27	7	10	14	21	*4	12
Protein Precipitin Titre. 1–500	+±	Crisis.	++	++	++	Crisis.	++	++	+++	+++
1–1000	++		++	++	++		+±	+±	+++	+++
1–2000	+±		+±	–	+±		+±	–	+±	+±
1–4000	–		–	–	+±		+±	–	++	+
1–8000	–		–		–		–		–	–
1–16000			–		–		–		–	–
Skin test.	neg.		pos.	pos.	neg.		pos.	pos.	pos.	pos.

* Day of crisis.

Precipitin Tests:
+++ = precipitate at bottom of tube with faintly cloudy supernatant fluid.
++ = slight precipitate with cloudy supernatant fluid.
+ = cloudy fluid.
± = faintly cloudy fluid.
– = completely negative.
0 = not done.

Skin Tests:
pos. = Typical delayed reaction greater than 1 cm. in diameter.
? = Reaction 0.5 – 1.0 cm. in diameter.
neg. = No reaction.
0 = Not done.

obtained with the carbohydrates. Of twelve patients tested while acutely ill, all failed to react. In eight individuals repeated injections were made both before and after recovery. Seven of them gave positive tests after crisis, and also with each repetition of the test during convalescence. These patients have not been retested since discharge from the hospital.

A test, when positive, developed slowly as compared with the reaction elicited by the carbohydrates. In 6 to 8 hours there appeared a small, dark red, circumscribed lesion, about 0.5 cm. in diameter. It gradually increased in size until a maximum was reached in 18 to 24 hours. Readings were recorded at this time. (See Fig. 3.) Regression which began after 36 to 48 hours, usually proceded slowly, and 3 to 4 days often were required for complete disappearance. When at its height, the lesion varied from 1 to 5 cm. in diameter; it consisted of a small central papule, dark red in color, surrounded by a bright pink erythema; the edges faded gradually into normal skin; slight tenderness was some times present and the local temperature often seemed to be increased. The protein reactions were similar in many respects to the tuberculin response. That this type of reaction, in contrast to the response induced by polysaccharides, was unrelated to the type of pneumococcus causing infection is borne out by the fact that of the 7 patients, in whom positive tests were obtained, 2 had Type I pneumonia, 2 Type II, 2 Atypical Type II, and 1 Group IV.

In determining the presence of pneumococcus protein precipitins, it was found that the sera of all the patients possessed antibodies reactive with this test material. The titre ranged from 1:2,000 to 1:16,000 in the different cases. However, in a single individual, variations in amount of precipitins occurring coincident with the progress of the disease were not sufficiently striking nor constant to be considered of significance. In Table II, the results of the protein skin tests are given for those cases in which repeated injections were made.

Table II shows the relation of the development of skin sensitivity both to the time of recovery from infection and to the titre of antiprotein antibodies with serum. From the data it appears that the protein sensitiveness develops after crisis, but, as contrasted with the

reaction to the carbohydrate, is unrelated to the quantity of precipitins present in the circulation.

The presence of antibodies to pneumococcus protein in the serum of human beings, has not, as a search of the literature reveals, been the subject of previous investigation. The possible significance of their occurrence is a part of this, as yet uncompleted, study. In addition to the cases cited above, the sera of 13 other pneumonia patients have been tested at frequent intervals after admission to the hospital until discharge. They were all found to possess in their serum protein precipitins in concentrations comparable to those recorded in Table II. The study of the anti-protein antibodies is being continued and a more complete report will be made in a later communication.

DISCUSSION

Although the observations reported in this paper on a relatively small number of patients represent an investigation which is, as yet, incomplete, sufficiently definite results have been obtained to warrant presentation. It has been shown that pneumococcus polysaccharides, when injected intradermally into pneumonia patients after crisis, can bring about in some instances, a local reaction. The cutaneous response, when obtained, was always elicited by the carbohydrate homologous to the type of pneumococcus causing infection in the patient. A patient's capacity to react became manifest coincident with recovery from infection. Furthermore, skin sensitivity has been found to parallel closely the presence of circulating type specific antibodies. The two phenomena have been found to appear at about the same time, to persist for a similar period, and to disappear at about the same stage of convalescence. However, the mere presence of type specific antibodies in the circulation is not the only factor necessary for the excitation of a skin response. The fatal case complicated by pneumococcus meningitis possessed type specific agglutinins in high titre but at no time could a definite skin response be obtained. Further investigation on a larger number of cases is necessary before the underlying mechanism can be fully interpreted. However, undetermined as the problem at the present may be, it is an interesting fact that these bacterial sugars, *protein-free*, and belonging

obtained with the carbohydrates. Of twelve patients tested while acutely ill, all failed to react. In eight individuals repeated injections were made both before and after recovery. Seven of them gave positive tests after crisis, and also with each repetition of the test during convalescence. These patients have not been retested since discharge from the hospital.

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to that group of immunologically specific substances known as haptens, are capable of producing a reaction in the skin of convalescents from pneumococcus pneumonia. The character of the skin response incited by the polysaccharides is unique in that it is urticarial-like in appearance and runs its course in 1 to 2 hours.

The reaction caused by pneumococcus protein, on the other hand, is similar both in appearance and evolution to that evoked by tuberculin. The protein reactions, when positive, reached the maximum intensity about 24 hours after injection and some times required 3 to 4 days to subside completely. Furthermore, a patient's capacity to react to protein has no relation to the type of pneumococcus causing the infection. Sufficient observations on the presence and quantitative variations of anti-protein antibodies have not yet been made to justify final conclusions. In the instances reported in this paper, antiprotein precipitins were found in all cases both before and after crisis. In any single individual the titre did not markedly vary during the course of disease, nor did it appear to influence skin sensitivity.

The data presented in this paper represents observations made in the course of lobar pneumonia. Similar tests on normal individuals and patients suffering from other diseases are being carried out and the results will be reported in a subsequent paper.

CONCLUSIONS

I. a. Pneumococcus polysaccharides, when injected intradermally into patients convalescent from pneumonia, are capable of eliciting a response. The polysaccharide inducing a cutaneous reaction was found always to be homologous in type to that of the pneumococcus causing the infection.

b. The character of the reaction incited by the protein-free bacterial sugars is of the immediate wheal and erythema type.

c. A patient's capacity to react was found to be intimately associated both with recovery from infection and with the presence of type specific antibodies in the circulating blood.

II. a. The so-called nucleo-protein of pneumococcus, when injected intradermally, also causes a local cutaneous reaction in patients during convalescence from lobar pneumonia.
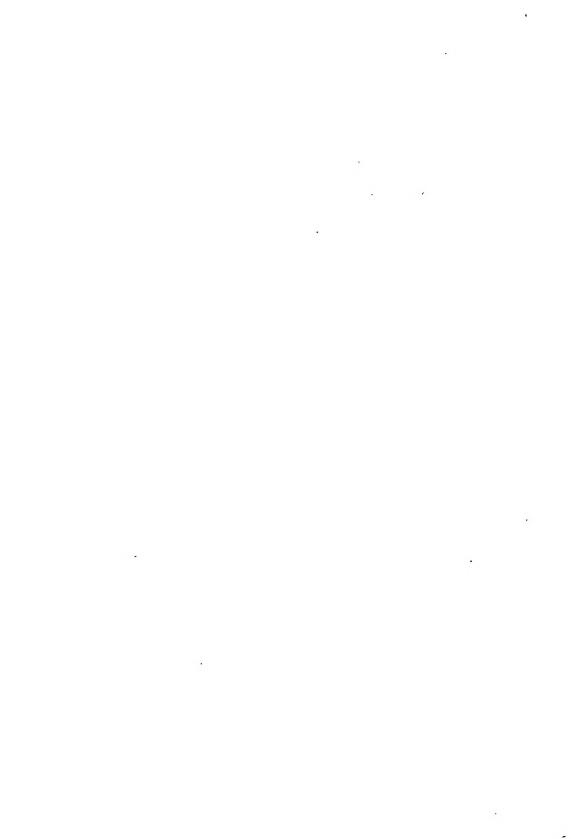
b. The local lesion resulting from the injection of protein is tuberculin-like in character, and differs from that evoked by the type-specific polysaccharides in gross appearance, time of development, and duration.

c. Individuals, acutely ill with and convalescent from pneumococcus pneumonia, possess in their circulating blood, precipitins reactive with pneumococcus protein. In the observations recorded, the concentration of anti-protein antibodies in the blood serum did not seem to influence the patient's capacity to react to intradermal injection of the protein.

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EXPLANATION OF PLATE 32

FIG. 1. Cutaneous reaction 30 minutes after the injection of 0.01 mgm. of Type I polysaccharide in a patient convalescent from Type I pneumococcus pneumonia. This illustrates the immediate wheal and erythema response to pneumococcus polysaccharide. Specific substances of Types II and III, and salt solution are negative. Roman numerals I, II, and III, designate the site of injection of Types I, II, and III carbohydrates; C represents the site of injection of salt solution. One-half natural size.

FIG. 2. Cutaneous reaction 30 minutes after the injection of 0.01 mgm. of Type II polysaccharide in a patient convalescent from Type II pneumococcus pneumonia. Specific substances of Types I and III, and salt solution are negative. Roman numerals and C serve the same purpose as in Fig. 1. One-half natural size.

FIG. 3. Cutaneous reaction 24 hours after injection produced by 0.01 mgm. of pneumococcus protein. This illustrates the delayed tuberculin-like response to pneumococcus protein. Four-fifths natural size.

POSSIBILITY OF HEREDITARY TRANSMISSION OF YELLOW FEVER VIRUS BY AEDES AEGYPTI (LINN.)

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Hereditary passage of yellow fever virus through mosquito eggs was first investigated by Marchoux and Simond (1906) who reported successful transmission to a human being through the first generation offspring of an infective mosquito. Rosenau and Goldberger failed to confirm this work; in fact none of thirteen susceptible human beings, bitten by mosquitoes reared from eggs of infected female stegomyia, showed any signs of yellow fever. Stokes, Bauer and Hudson (1928) also obtained negative results when they allowed 79 mosquitoes, reared from the eggs of infective females, to bite a normal *rhesus* monkey. Hindle (1929) has pointed out the importance of further experimentation, especially as he has shown by inoculation of various parts of infected mosquitoes that the virus is not confined to the salivary glands. It was felt by the West African Yellow Fever Commission that information on this important point was still inadequate, and at the suggestion of Dr. Henry Beeuwkes, the problem was again taken up.

EXPERIMENTAL

Two methods were employed, direct inoculation into test animals of an emulsion of eggs laid by infective mosquitoes, and transmission tests with adults reared from such eggs. Since it has been shown by injection (Bauer and Hudson, 1928) that yellow fever virus is present in infectious form in adult mosquitoes at all times subsequent to their infecting blood-meal, it seems logical to assume that, if virus is present in the ova, it could be detected by similar injection procedures. This seems especially likely when large numbers of ova are employed as in the experiments here reported.

A group of 172 normal females of *Aedes aegypti* was fed on an infected monkey during the initial period of fever (Asibi strain of virus). These mosquitoes were subsequently shown to be infective, since all normal monkeys bitten by them died of typical yellow fever. The mosquitoes were then segregated and 65 males added to obtain fertile eggs. At desired intervals ova were collected on strips of continuously wet blotting paper. The ova were never allowed to dry. All were used within two to three days and the majority within twenty-four hours after oviposition. Batches of eggs were removed after the first or infecting blood-meal, and after the second, fourth and fifth testing (normal) meal, that is 14, 41 and 54 days after the first feeding. Of the first two batches of ova, one half were employed for direct injection into test animals, and the other half placed in water to obtain adults for subsequent feeding tests. As the original lot of mosquitoes laid an insufficient number of eggs after the fourth blood-meal, they were given a fifth meal for the rearing of adults to be used in additional biting experiments.

It is known that in certain diseases (Rocky Mountain spotted fever particularly), there is an "activation" of the etiologic agent following a blood-meal of the maturing arthropod host. For that reason, more than one feeding of blood was allowed for each lot of mosquitoes, both parent and offspring. After the first feeding of each batch there was allowed a period of time at least as long as the incubation period (8 days or more). This gave a check on the relationship of both time and number of blood-meals of parents and progeny to the problem in hand.

Experiment I

A. *Eggs.* Approximately 400 eggs, laid soon after the first infecting feeding, were washed off of the blotting paper into 50 c.c. normal saline and gently centrifuged. Three c.c. of the supernatant fluid were drawn off and injected into normal *M. rhesus* C; the remainder of the liquid was discarded. The animal registered normal temperatures for 15 days. It was then found susceptible by the injection of known virulent blood, and, during the febrile attack which resulted, was killed as a source of virus for other experiments.

After centrifugalizing, the ova were drained, ground in a sterile mortar with neutralized glass powder, diluted with 5 c.c. normal saline, and 2.5 c.c. of the resulting mixture injected into each of two normal monkeys, A and B. No reaction followed for a period of 15 days and both died of yellow fever when tested later for susceptibility.

B. *Adults.* The second half of the eggs were immersed while still wet and the adult females that emerged, designated as Lot 185, were used in the following three tests:

1. A normal *rhesus* (D), was bitten by 254 insects of this hatching. No apparent reaction occurred over a period of 19 days, nor was any noted when it was tested for susceptibility with known infectious material. In the light of the subsequent experiments, it seems probable that this animal was insusceptible to yellow fever rather than immunized by the bites of the mosquitoes.

2. Fourteen days after they had been fed on Monkey D, 110 specimens of the same lot were allowed to bite another normal monkey, (E). This animal continued to show normal temperatures for a period of 17 days. It was then inoculated with proved infectious blood, but although a sharp febrile reaction resulted, the monkey recovered.

3. Forty-two days after the first feeding, the 96 insects remaining alive were stupefied with tobacco smoke and macerated in 4 c.c. of normal saline. The suspension was then injected subcutaneously into normal *M. rhesus* F. No reaction was noted during a period of 17 days in this animal which when later exposed to the bites of an infective lot of mosquitoes, died of typical yellow fever.

Experiment II

A. *Eggs.* The parent lot of mosquitoes was given a second blood-meal on a normal animal 14 days after their infecting blood-meal. Approximately 400 ova were collected and treated exactly as in Experiment I. Injection of supernatant fluid after concentration in the centrifuge was omitted in this and in the following experiments. Two normal monkeys, G and H, were injected with 2.5 c.c. each of the egg emulsion, as before. The animals remained normal for 15 days and then proved susceptible to inoculation of virulent blood, developing fatal yellow fever.

B. *Adults.* After removal of enough for use in section A of this experiment the remainder of the eggs of the second laying by the original mosquito lot was immediately immersed for hatching without drying. The adult females that emerged were labelled Lot 187 and employed in the two following tests:

1. A normal monkey (I) was bitten by 350 insects of this lot 38 days after the original infecting meal of the parent lot. After a period of 22 days, the animal still remained normal. It died with characteristic lesions following inoculation of virulent blood.

2. Two hundred and fifty *A. aegypti* of the same lot fed on a second normal *rhesus* (J) 14 days after the first test. No reaction occurred during the following 3 weeks and, when tested for susceptibility with infectious blood, this animal succumbed to yellow fever.

Experiment III

A. *Eggs.* Ova were again collected after the fourth blood-meal of the parent lot of aedes on a normal monkey, 41 days from the time of their initial feeding. About 400 eggs were washed, concentrated and ground in a sterile mortar without the use of abrasive material. The mixture was then diluted to 5 c.c. with normal saline and divided for injection into two normal animals (K and L) as in the previous experiments. No reaction was noted during the succeeding 17 days and both monkeys died following inoculation of yellow fever virus.

B. *Adults.* As already explained a fifth blood-meal for the parent lot of mosquitoes was necessary to obtain eggs for the last experiment. This blood-meal occurred 54 days from the time of the original infecting feeding. The ova

collected were immediately immersed for hatching. The adult females obtained were designated as Lot 204 and employed in the two following tests:

1. Normal *M. rhesus* M was bitten by 44 specimens of this lot. It showed no reaction during a period of 24 days and was not susceptible when tested with

TABLE I

Results of Attempts to Transmit Yellow Fever Virus to Normal M. rhesus through First Generation A. aegypti from Infective Parents

Experiment No.	Rhe-sus	Date	Exposure	Results	Susceptibility test
I Eggs and adults obtained after 1st blood-meal of parent lot, Jan. 24	A	Jan. 28	2.5 c.c. saline emulsion of 200 eggs	No reaction for 15 days	Died, yellow fever
	B	do	do	do	do
	C	do	3.0 c.c. saline used for washing above eggs	do	High fever, killed for virus
	D	Mar. 2	Bitten by 254 reared females, Lot 185	No reaction for 19 days	Insusceptible
	E	Mar. 16	Bitten by 110 of above lot	No reaction for 17 days	Fever, recovered
	F	Apr. 13	Injected with 96 of above lot	do	Died, yellow fever
II Eggs and adults obtained after 2nd blood-meal of parent lot, Feb. 7	G	Feb. 12	2.5 c.c. saline emulsion of 200 eggs	No reaction for 15 days	Died, yellow fever
	H	do	do	do	do
	I	Mar. 11	Bitten by 350 reared females, Lot 186	No reaction for 22 days	do
	J	Mar. 25	Bitten by 250 of same lot	No reaction for 21 days	do
III Eggs and adults obtained after 4th and 5th blood-meals of parent lot, Mar. 5 and 18	K	Mar. 14	2.5 c.c. saline emulsion of 200 eggs	No reaction for 19 days	Died, yellow fever
	L	do	do	do	do
	M	Apr. 22	Bitten by 44 reared females, Lot 204	No reaction for 24 days	Insusceptible
	N	May 1	Injected with 41 of same lot	No reaction for 15 days	Died, yellow fever

known infectious blood. This was the second insusceptible animal encountered during these experiments.

2. Nine days from the time of the first blood-meal 41 insects in Lot 204 remained alive. These were stupefied with tobacco smoke, macerated in 3 c.c.

normal saline in a sterile mortar and injected into a normal *rhesus* (N) which showed no reaction during the following 15 days. It developed fatal yellow fever when later tested for susceptibility. This test was made 63 days after the original infecting blood-meal of the parent lot of mosquitoes.

In section A of Experiments I and II, neutral glass powder was used as an abrasive aid in pulverizing the chorion of the eggs. In sections B of Experiment I, and in A and B of Experiment III, the eggs and adults were ground up by friction alone. At no time were the ova used in the above experiments allowed to dry. All injections were made subcutaneously and carried out immediately following maceration thus eliminating possible deleterious effects from standing in suspension. It therefore seems unlikely that any explanation other than absence of the virus can account for negative results following the various injections.

These results are in agreement with those of Stokes, Bauer and Hudson (1928). The data are summarized in Table I.

SUMMARY AND CONCLUSIONS

Attempts to obtain passage of yellow fever virus from one generation to the next in *A. aegypti* were unsuccessful. Subcutaneous injections at varying intervals of a saline emulsion of 200 eggs laid by an infective lot of mosquitoes produced no reaction in six normal *M. rhesus* monkeys. Negative results were also obtained in five biting and two injection experiments with progeny of the same infective lot of mosquitoes in which seven normal monkeys were used. The eggs consisted of batches laid after the first, second and fourth blood-meals of the original lot; the latter feeding occurred 41 days after the initial infecting meal. The imaginal offspring represented rearings following the first, second and fifth blood-meals of the parent lot. The last feeding occurred 54 days after the first.

It is concluded that under the conditions of the experiments here reported hereditary transmission of yellow fever by *A. aegypti* is improbable. Variations in age and in number of blood-meals of parent and offspring mosquitoes had no effect in achieving passage of the virus from one stage of the insect to another.

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NUMBER OF GLOMERULI IN KIDNEY OF ADULT WHITE RAT UNILATERALLY NEPHRECTOMIZED IN EARLY LIFE

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Despite many investigations, the total number of glomeruli in the hypertrophic kidney remains a question. The problem may be approached in two ways; first, experimental production of a hypertrophic kidney by unilateral nephrectomy, and second, observations of the lesion in congenital anomalies of the urinary tract. The present paper reports the results of experimental investigations in the white rat. In a future paper, observations on congenitally asymmetrical kidneys will be presented.

Two methods have been used by other investigators in the enumeration of the glomeruli in the hypertrophic kidney, a relative and an absolute method. The relative method compares the total glomeruli in equal areas of standard sections. The absolute method consists in a complete enumeration of the glomeruli. Three types of the absolute method have been developed. Kittleson (1) counted the glomeruli in serial sections and by the ingenious use of carbon paper avoided duplication. This method is applicable to small kidneys, such as the rat and mouse, but is too laborious for larger kidneys. Two students of Bensley have evolved injection methods which are quite accurate, yet are not time consuming. Nelson (2) used Janus Green B as a vital dye and counted the whole glomerulus in teased preparations. Vimtrup (3) modified Traut's (4) Prussian blue injection method and enumerated the glomeruli in the kidneys of man, dog, cat, and white rat with a high degree of accuracy.

The general trend of previous investigation separates the problem into two classes, the response of young and of adult animals. There is general agreement that unilateral nephrectomy in the adult animal has no effect on the total glomeruli in the opposite kidney. The reported results in young animals are at variance.

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Tizzoni and Pisenti (5), Lorenz (6), Galeotti and Villa-Santa (7) and Zanetti (8) found an increase, while von Gudden (9), Ribbert (10), Debenedetti (11) and Peruzzi (12) were unable to find histologic or quantitative evidence of the formation of new glomeruli. More recently Arataki (13) has demonstrated by the accurate method of Kittleson (1) that there is no increase of total glomeruli in the white rat after unilateral nephrectomy at 20 to 50 days of age. Jackson & Shiels (14) in one animal found no increase after operation at 7 days of age.

In the rat, there is an active formation of new glomeruli for some days after birth and it seemed desirable to extend these accurate studies to include animals operated at a time when the kidney exhibits definite nephrogenesis.

Method

Litters from thoroughly tame females were selected. In all cases one-half were operated and one half saved as controls. The right kidney was removed through a lumbar incision under ether anaesthesia. The pedicle was tied by one ligature and the wound closed by catgut and celloidin.

The mortality of animals operated at 1 day of age was high, only 1 of 10 surviving to adult life, while the mortality at 3 days of age was about 50 per cent.

At about 165 days of age, the animals were killed by a blow on the head and the kidneys injected with Janus Green B and counted according to the technique of Nelson (2). In all instances the counts represent a complete enumeration of the glomeruli in the left kidney.

RESULTS

Inspection of the accompanying table reveals that, aside from biological variation, the experimental animals do not differ in any marked respect from the controls.

When the results are submitted to statistical analysis, the results leave much to be desired.

	Control		Experimental
Arithmetical mean	20,162		20,371
Difference		209	
Standard deviation	1,184		1,480
Probable error	326		446
Probable error of difference		552	

The finding of a probable error of the difference twice as great as the actual difference makes the results ambiguous and points to insufficient number of observations. Assuming that the difference of the arith-

metical mean from the individual observations remains the same, a study of 50 animals in each group would be necessary in order to bring the probable error of the difference to about the same figure as the actual difference. It is clearly impossible to undertake such a study in one laboratory even if the mortality of unilateral nephrectomy in young animals were low.

Despite the failure of mathematical proof, we believe that the results here reported demonstrate that unilateral nephrectomy in the

TABLE I

Rat No.	Type	Sex	Body wt.	Kidney No.	Total count
Nephrectomy—1 Day of Age—Sacrificed—165 Days of Age					
			gm.		
1	Control	Male	370	144	18,562
2	Experimental	Male	390	111	18,389
Nephrectomy—3 Days of Age—Sacrificed—166 Days of Age					
3	Control	Male	310	113	21,411
4	Experimental	Male	285	112	18,887
5	Experimental	Male	300	115	21,540
Nephrectomy—3 Days of Age—Sacrificed—168 Days of Age					
6	Control	Male	290	127	21,890
7	Control	Male	280	130	20,384
8	Control	Male	325	132	19,188
9	Control	Male	320	134	19,526
10	Experimental	Male	360	128	20,881
11	Experimental	Male	265	129	22,159

The animals in each group are litter mates.

young white rat has no effect on the total number of glomeruli which will be formed in the opposite kidney. Certainly, the hypertrophic kidney does not contain double the number of glomeruli observed in the normal kidney.

The counts here reported for the white rat differ appreciably from those of Kittleson (1), Arataki (13) and Vimtrup (3). The latter counts were made on the Wistar rat and average 28,000 to 30,000. We have been able to duplicate these counts on the standard rat, but in our colony of black and white animals have never secured a count

higher than 22,200. We are at present attempting to explain this difference on a sub-species difference and will report the results of counts on hybrids at a future date.

CONCLUSION

Unilateral nephrectomy during the period of active nephrogenesis in the white rat has no effect on the total number of glomeruli which will be formed in the opposite kidney.

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THE FILTRABILITY OF YELLOW FEVER VIRUS AS EXISTING IN THE MOSQUITO*

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Evidence is lacking that yellow fever virus procured from the mosquito vector, *Aëdes aegypti*, differs from that procured from man or monkey, except possibly with respect to filtrability. Stokes, Bauer, and Hudson[1] found that the virus in the serum of infected monkeys could be filtered readily through Berkefeld filters V and N, but that the virus from infective mosquitoes, ground and suspended in salt solution, could not be passed through these filters. The experiments here described were undertaken to determine whether the observed difference in filtrability was due to different properties of the virus itself or to differences in the medium in which it was contained. The filtrability of the virus from mosquitoes was tested with specimens obtained during the incubation period, meaning thereby the period before the bites of the insect become infective, as well as later, for Bauer and Hudson[2] had shown that the virus is present in the mosquito during the entire incubation period in such quantity and in such state as to be infective to monkeys when inoculated experimentally.

Various investigators have observed that the filtrability of minute particles, and even of dissolved substances, is affected by their electrical charge, and that the charge may be altered by changing the hydrogen ion concentration of the fluid in which they are contained. The surface tension of the fluid, through its relation to interfacial tensions within the filter during filtration, appears to be another of the

[1] Stokes, A., Bauer, J. H., and Hudson, N. P., *Am. J. Trop. Med.*, 1928, 8, 103.
[2] Bauer, J. H., and Hudson, N. P., *J. Exp. Med.*, 1928, 48, 147.

properties which affect the filtrability of suspended or dissolved substances. The evidence with regard to these factors and others influencing adsorption in filters has been summarized by Mudd.[3]

Methods

The mosquitoes used in these experiments were of the species *Aëdes* (*Stegomyia*) *aegypti*, and all were bred in the laboratory. The four mosquitoes used during the incubation period in Experiment II were descended from mosquito ova sent us from Edenton, North Carolina, by Dr. Mark F. Boyd. The others were descendants of mosquitoes from ova brought to us from Bahia, Brazil, by Dr. J. H. Bauer.

The newly emerged imagoes were placed in cylindrical glass catching tubes, one male and one female in each, and the tubes were closed with a layer of marquisette bound firmly in place with adhesive tape. The mosquitoes were fed with moistened raisins laid on the marquisette. When feedings on monkeys were required by the experiments, the animals were anesthetized by intraperitoneal injections of iso-amyl-ethyl-barbituric acid and the mosquito tubes were held against the skin so that the insects could bite through the meshes of the cloth.

The mosquitoes used in these experiments were infected by feeding them on monkeys, experimentally infected with yellow fever, on the first day of fever (40°C. or over). Of the 16 mosquitoes used in Experiment I, 15 had fed on one or other of two monkeys inoculated with the French strain of yellow fever from West Africa, and one had fed 22 days before on a monkey inoculated with the F. W. strain of virus from Brazil. The F. W. strain had been obtained originally from Dr. H. de Beaurepaire Aragão of the Oswaldo Cruz Institute in Rio de Janeiro. The mosquitoes used in Experiment II had been fed on monkeys which had been inoculated with the Asibi strain of virus from West Africa. The test inoculations noted in the tables were made with dried virus of the Asibi strain. The origins of these strains of virus have already been published.[1, 4, 5]

To obtain material for filtration the mosquitoes were stupefied with tobacco smoke, removed from their containers, and finely ground up in the appropriate fluid in a mortar.

The monkeys used in these experiments were of the species *Macacus rhesus* except Monkeys 1 and 2 in Experiment I, which were *Macacus cynomolgus*. They were inoculated by intraperitoneal injection or were bitten by mosquitoes. Rectal temperatures of the monkeys were taken twice each day. In the cases of those which contracted yellow fever the diagnosis was confirmed by necropsy and histological examination of the tissues.

The filters used were Berkefeld V or N, of size 1.5 by 6.0 cm. They were tested under water by air pressure before and after use, and no defects were found. To

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[3] Mudd, S., in Filterable Viruses, edited by Rivers, T. M., Baltimore, 1928, 55.

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each fluid to be filtered was added a heavy suspension of *B. prodigiosus* (1.0 cc. in Experiment I and 0.5 cc. in Experiment II), and in no case did this organism appear in the filtrate. The fluids passed through the filters in a very few minutes. We were unable to maintain a constant pressure with the apparatus used, and the negative pressure started low and rose during filtration. It reached 61 cm. of mercury in the filtration with the Berkefeld filter V in Experiment I, but in the other filtrations it did not reach 54 cm., the least negative pressure measurable by the manometer used.

The serum used in the experiments was obtained by bleeding a normal monkey from the heart under ether anesthesia on the day before the first experiment. An equal volume of 0.9% sodium chloride solution was added to the serum in each experiment. In the first experiment the mosquitoes were ground up in salt solution and the resulting suspension was divided and incorporated in the fluids used in the three parts of the experiment. In the second experiment the mosquitoes were ground in the serum-saline mixtures.

The hydrogen ion concentrations of the saline solution and of the undiluted serum were determined before filtration and found to be 6.6 and 7.8 respectively. The figures in the table show the *pH* of the filtrates.

Measurements of the surface tension of the monkey serum used in both experiments when diluted with an equal part of 0.9% sodium chloride, were made with the du Noüy tensiometer at the time of the second experiment. The serum-saline mixture gave a reading of approximately 55 dynes at 20°C. when corrected for temperature. At the same temperature the sodium chloride solution would have a corresponding surface tension of 77 dynes.

Experiment I

The purposes of Experiment I were to determine whether yellow fever virus of infective mosquitoes would pass Berkefeld filters when placed in monkey serum, the fluid in which the virus from monkeys had been shown to be filtrable, and further to test the filtrability of the virus when in saline solution. The results are shown in Table I.

Sixteen mosquitoes were used. The intervals since they had fed on infected monkeys were 14 days for 3 mosquitoes, 16 days for 12 mosquitoes, and 22 days for one. It was expected that all had become infective, as the temperature of the mosquito room stood as a rule between 26° and 28°C. and was controlled by automatic electric heaters, and was humidified.

The suspension of mosquitoes in salt solution was filtered through the coarser filter, Berkefeld V, as it was not expected that the virus would pass through because of the results of earlier workers.[1] The suspension in diluted serum was filtered through a finer filter, Berkefeld N, as it seemed probable that the virus would pass through, and it was desired to make the test as rigid as possible. As will be seen in Table I, the serum filtrate contained enough virus to induce yellow fever in a

monkey, the incubation period being only 2.5 days (maximum temperature 39.8°C.), with death on the fifth day after inoculation. The salt solution filtrate from the coarser filter produced no effect and the monkey remained susceptible to yellow fever.

The experiment showed that the virus in infectious mosquitoes is filtrable when suspended in diluted monkey serum; while the failure

TABLE I

Experiment I. Filtrability of Yellow Fever Virus in Infective Mosquitoes When Suspended in Salt Solution and When Suspended in Diluted Normal Monkey Serum. Performed March 16, 1929

Number of monkey inoculated	Grade of Berke-feld filter	Material injected into monkey	pH of filtrate	Amount injected	Results	Date of inoculation to test suscepti-bility	Result of test inoculation
				cc.			
1	V	Filtrate from infective mosquitoes in salt solution	6.4	10*	No reaction	April 20	Died April 26 of yellow fever
2	N	Filtrate from infective mosquitoes in diluted normal monkey serum	7.7	10*	Died, March 21 of yellow fever		
3		Unfiltered infective mosquitoes in salt solution		2**	No reaction	April 13	Died April 17 of yellow fever

* Contained the equivalent of five mosquitoes.
** Contained the equivalent of one mosquito.

to filter the mosquito virus suspended in saline solution was devoid of significance, since the control monkey was not infected or immunized by the injection of a small amount of the unfiltered suspension. It seems probable that some factor in the artificial environment of the mosquitoes had not permitted the virus in their bodies to become or remain abundant. That this was the case is suggested also by the fact that in two additional experiments, mosquitoes which had fed on

infected monkeys on the first day of fever did not produce infection or immunity when injected into normal monkeys.

In the unsuccessful experiments referred to, an attempt was made to determine whether the virus would be filtrable in sodium chloride solution and also in infusion broth if the pH was maintained by a phosphate buffer at 7.8, the pH at which the virus in the mosquito-serum mixture in Experiment I had been shown to be filtrable.

TABLE II

Experiment II. Filtrability of Yellow Fever Virus in Infective Mosquitoes and in Mosquitoes during the Incubation Period, When Suspended in Diluted Normal Monkey Serum. Performed June 17, 1929

Number of monkey inoculated	Grade of Berkefeld filter	Material injected into monkey	Time since mosquitoes had bitten infectious monkey	pH of filtrate	Amount injected	Result
			days		*cc.*	
4	N	Filtrate from 2 mosquitoes in diluted normal monkey serum	11	7.2	11.3*	Died June 22 of yellow fever
5	N	Filtrate from 4 mosquiotes in diluted normal monkey serum	6	7.2	10.3**	Died June 23 of yellow fever
6	Bitten by one of the 11-day mosquitoes and all of the 6-day mosquitoes on the day of the experiment***					Died June 22 of yellow fever

* Contained the equivalent of 1.5 infective mosquitoes.

** Contained the equivalent of 2.7 mosquitoes in the incubation period.

*** Infected by the 11-day mosquito, as the others were in about the middle of the incubation period of the virus.

Experiment II

The second experiment was devised to test again the filtrability of the virus from infective mosquitoes, as determined in Experiment I, and to ascertain whether the virus in the mosquito is filtrable also during the incubation period. By the use of the term "incubation period" we do not intend to imply that there occurs an increase in the total amount of virus in the mosquito during this period, for that has not been proven. The results of Experiment II are shown in Table II.

This experiment was carried out in the early summer and the environmental conditions may have been better for the survival or increase of the virus in the mosquitoes. The temperatures in the mosquito room were higher than before and frequently rose above 30°C. Another difficulty developed, however,—a high mortality among the mosquitoes. It was possible, nevertheless, to carry out Experiment II with the few survivors, as the virus content of the mosquitoes proved to be great.

Of one lot of mosquitoes only two females remained alive on the eleventh day after biting an infected monkey. To conserve the insects for filtration, the test for infectivity was made by applying the live mosquitoes to a normal monkey instead of using some of the unfiltered mosquito suspension for inoculation. Only one of the mosquitoes bit the animal, but that was sufficient to infect. The virus from the two mosquitoes mentioned, after filtration in diluted serum through a Berkefeld N filter, infected a monkey on inoculation. The incubation period was 2.5 days and death occurred on the fifth day. This confirmed the previous observation with regard to virus from infective mosquitoes when suspended in diluted serum.

In the second part of the experiment four mosquitoes were used which were in about the middle of the virus incubation period. They had fed on an infected monkey only six days before. At first it was hoped to keep them longer and they were fed on the normal monkey (Number 6), before the two mosquitoes above referred to were applied to it, in the hope of prolonging their lives. Some of them appeared to be dying in the afternoon of the same day, and they were used at once. When suspended in diluted serum the virus from these mosquitoes, though in the incubation period, passed a Berkefeld N filter, and infected a monkey, which came down with yellow fever in 3.5 days after inoculation and died on the sixth day.

CONCLUSIONS

The virus of yellow fever as it exists in *Aëdes aegypti* mosquitoes, both in their so-called infective stage and in the intermediate condition termed the "incubation period," is capable of passing through Berkefeld N filters when suspended in normal monkey serum, although earlier investigators have shown that the virus from infective mosquitoes will not do so when suspended in physiological salt solution.

The virus of yellow fever as it exists in mosquitoes behaves with regard to filtration through Berkefeld N filters as does the virus in the blood of infective monkeys.

THE BLOOD IN HOG CHOLERA*

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The present study concerns the properties of the blood of the cholera animal considered as the carrier of the virus of the disease on the one hand, and as reflecting the pathological effects of the disease on the other.

If the blood of the cholera hog be drawn with aseptic precautions in the early days of the fever (before the 5th) it will frequently be found free of bacteria demonstrable by the usual methods of aerobic and anerobic cultivation. Inoculated into a normal animal the disease may be readily produced after an incubation period varying from 3 days to 3 weeks depending on the activity of the virus and the resistance of the animal.

The virus is both free in the serum or plasma and, as Dinwiddie (1) showed, so associated with the cells that repeated washings with the centrifuge do not detach it. Although this observation is usualy quoted as relative to red cells, Dinwiddie's paper does not discriminate between the red and white cells and blood platelets. Dinwiddie also noted round, stainable bodies in the red corpuscles (11), while King, Baeslack, and Hoffman (2) believed that they found a small spirochete in small numbers in the blood.

The actual potency of the virus has quite recently been determined quantitatively. Uhlenhuth (4) had stated that dropping a minute amount of virus into the uninjured conjunctival sac sufficed to produce infection but gave no actual measures. The reports of McBryde and his coworkers, however, give increasingly accurate determinations of blood virulence which their latest figure places at 0.00003 cc. (5). Roderick and Schalk (6) found that 0.00002 cc. of the virus serum was sufficient to produce an infection with hog cholera.

EXPERIMENTAL

The work here reported was done with a strain of virus obtained from a commercial laboratory in 1925. It has been maintained by occasional passage through swine at this laboratory. Our experimental animals are bred in the Institute's establishment. They are fed no animal products, and are cared for with every attention to isolation. They have never been vaccinated against hog cholera, and there has been no case of the disease in the stock since its establishment as a segregated herd in 1920. No animal inoculated with usual quantities of the virus strain has failed to develop the disease when kept under the usual conditions.

In the early passages intraperitoneal inoculation with 1, 2, or 4 cc. of virus blood taken about the 5th day of the fever quite regularly resulted in the establishment of disease after an incubation period of 4 to 7 days. Recently the incubation period has been quite regularly 3 days. Experiment has shown that our virus is approximately as active as that reported by McBryde. We have induced typical cholera with a four day incubation period with 0.00001 cc. of blood. The further tenfold dilution failed to infect.

Repeated examinations of blood have been conducted in an effort to obtain visual evidence of the virus. Bodies similar to those described by Dinwiddie are readily made out in variable numbers. Careful control has shown indistinguishable structures in normal swine blood. The larger bodies seem to be those frequently noted by hematologists as "Jolly bodies." The smaller seem to be associated with the occurrence of reticulation and basophilic granulation of the red blood corpuscles, with modifications in appearance due to the application of unusual or unstandardized staining methods.

Blood smears have been made in the usual way on carefully cleaned slides. They have been fixed with heat, methyl and ethyl alcohols, alcohol ether mixture, acetone, formaldehyde, Zenker's fluid or Regaud's fluid and stained after each fixation with Gram stain, Loeffler's methylene blue, carbol fuchsin, and Giemsa's stain. Many preparations have also been made with Borrell's toluidine blue,

with Goodpasture's fuchsin as for Negri bodies and with eosinmethylene blue. Fresh blood has been mounted on slides under cover slips, sealed and examined. In a number of instances the white cells have been concentrated before mounting and in other cases the serum or plasma, freed so far as possible of cellular elements by centrifugalization, has been examined. Fresh preparations have also been stained supravitally with neutral red and Janus green. Both direct and dark field illuminations have been used with the best available optical apparatus, on both the fresh and the fixed and stained blood. The examinations have been carried out with the aid of a mechanical stage, and the study of preparations has been both prolonged and systematic.

The examinations have shown the presence of small numbers of bacteria in the preparations from both supposedly normal and from diseased animals on several occasions.

No abnormal formed element has been observed which could by any possibility be identified with the virus. In special no spirochete was seen, and no motile form of any sort amongst the granular matter so prominent in dark field films prepared in the usual manner from blood, blood plasma and serous exudates. Furthermore when blood was thoroughly defibrinated by stirring with a wire whip and examined at once with the dark field the intercellular spaces were often almost optically empty. No cell inclusion peculiar to the disease has been found.

On the basis of a virus potency of 0.00001 cc. each preparation may be supposed to contain at least 1,000 elements. (The usual well prepared thin blood smear on a slide uses from 1–200 to 1–500 cc. of blood and a fresh preparation on a slide under a 7/8 inch cover for either dark field or direct illumination cannot hold more than 1/100 cc.) This imparts confidence that the opportunity for observing elements, were they demonstrable by the methods employed, is adequately afforded by the material.

Many and repeatedly renewed attempts have been made to cultivate the virus, all unsuccessful. Particularly it may be noted that the method successfully used by Marchoux (7), and later by Landsteiner and Berliner (8) for the propagation of the virus of "Hühnerpest" has been thoroughly tried for our purpose. Anaerobic as well as aerobic conditions have been used without result. We have not so far tried the symbiotic method with tissue cultures or other microorganisms.

The announced successful cultivation of the virus, in the form of *Spirocheta hyos*, by King and Drake (9) cannot be highly regarded when it is recognized that the dilution potency of the virus is so much greater than they supposed.

The Cellular Pathology of the Blood

In hog cholera it was noted by King and Wilson (3) that an average of a number of examinations showed a leucopenia of moderate degree. They found that for normal hogs the total leucocyte count averaged 19,982 (43 examinations on 42 individuals). The maximum recorded as normal was 39,296, the minimum was 10,070. For the cholera animals the total average was 15,515 (20 examinations of 16 individuals). The maximum was 23,600, the minimum 7,200. The authors give no data as to the method or materials of inoculation for the animals whose blood counts were given. No protocols appear in which the progress of the blood changes can be correlated with advancing disease and it is impossible to reconstruct such a correlation from the information given in the tables. Their average change is in the same direction as that which we are able to record, but with their tables alone one would conclude that the changes were much less significant in point of regularity and extent than those we have experienced.

Lewis, Shuler, McElroy and Ritter (10) confirmed the observations just mentioned but apparently did not greatly extend them. The lowest white cell count observed by these authors was 7,000 per cu. mm. Like King and Wilson, they found a progressive decrease in the red blood corpuscles and a comparable decrease in hemoglobin. Their data do not permit of a clear correlation between the development of the anemia and the progress of the disease.

Dinwiddie (11) found leucopenias in swine suffering from hog cholera that were very marked in some animals and corresponded very closely to what we have observed in the experiments to be reported here. It is impossible, however, from Dinwiddie's figures to reconstruct the actual progress of the leucopenia or to determine its possible significance as an aid to the identification of a disease as hog cholera. Regner (12) in Germany, has attempted to differentiate hog cholera, swine plague and swine erysipelas by means of the blood picture and has described a rapidly occurring leucopenia in acute hog cholera.

It appeared to us that if a definite leucopenia was indeed a constant feature of acute hog cholera it might be used as a discriminating aid to the diagnosis of the disease, and we have therefore restudied the question.

It became apparent that the usual method of collecting the blood for counts was ill suited to any considerable series of observations on swine. The task of filling the counting pipettes accurately from a

skin puncture was so difficult and time-consuming that a more satisfactory method was sought. The technique finally used in making several hundred blood counts on swine was so simple and rapid that it seems worth while to describe it in detail. With only slight modification it could be adapted for use with other large animals.

The equipment needed for the blood collection consists of a pair of moderately heavy scissors, a number of 50 ml. round-bottomed, wide-mouthed centrifuge tubes or ordinary wide vials with rubber stoppers, some small rubber bands, and a small amount of an anticoagulant. For the latter, we have used heparin, 1 mg. per 5 cc. of blood, in most of our work, but either potaassium oxalate, 2 mg. per cc. of blood, or sodium citrate, 2 mg. per cc. of blood, will be found satisfactory. The latter two anticoagulants may give rise to imperfectly formed crystals but these can be distinguished by the use of the "high dry" objective.

Blood is obtained by cutting off a small portion of the hog's tail with scissors, thus opening the relatively large coccygeal arteries, and is collected in a 50 ml. wide-mouthed tube containing powdered antigoagulant in sufficient amount. Our practice has been to keep in readiness a number of these tubes each containing sufficient anticoagulant for 5 cc. of blood, the 5 cc. level on the tube being marked. After the specimen has been obtained the tube is closed, using a rubber stopper preferably, and further hemorrhage from the animal's tail is prevented by wrapping a rubber band about it over a small pad of absorbent cotton, as near the tip as possible, rather tightly.

Before making a count on the blood the tube should be agitated again to insure thorough mixture of the corpuscles and plasma which sometimes separate rather rapidly on standing especially in blood from febrile animals. Then a small amount (0.5 cc. is sufficient) is removed from the tube by pipette and run out on a clean glass microscopic slide. From the pool thus formed the hemocytometer pipettes are promptly filled using 2 per cent acetic acid as the diluent for the white blood cells and physiological salt solution as the diluent for the red blood corpuscles. The blood film for the differential count can be made in the usual manner. From this point the counts are conducted after the standard methods.

The method has been checked against counts made on blood collected directly from skin puncture and found to agree satisfactorily. A few typical counts are presented in Table I. Animals with normal numbers of red and white cells and those with the anemia and leucopenia characteristic of hog cholera are included. We have also determined that counts made on samples of blood that have stood at warm room temperature all day are comparable to those made immediately after the blood is drawn (Table II). This makes it possible to study the blood of animals collected at a distance from the laboratory. Specimens are satisfactory for "supravital" staining with neutral red and Janus green for at least 3 hours. The leucocytes retain their motility and their cytoplasm takes up the dyes satisfactorily for this length of time at least.

TABLE I

Comparison of Counts from Counting Pipettes and from Tubes with Heparin, Potassium Oxalate or Sodium Citrate

Animal No.	Cells	Direct (ear)	Indirect (bled from tail)		
			Heparin (1 mg. per 5 cc. of blood)	Potassium oxalate (2 mg. per cc. of blood)	Sodium citrate (2 mg. per cc. of blood)
		per cu. mm.	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*
434	Leucocytes	3,200	2,320	2,900	2,480
	Erythrocytes	4,435,000	4,575,000	4,435,000	4,175,000
435	Leucocytes	6,440	6,520	6,400	6,300
	Erythrocytes	5,110,000	5,675,000	5,515,000	5,725,000
396	Leucocytes	24,750	27,400		
	Erythrocytes	7,162,500	7,037,500		
437	Leucocytes	22,040	22,600	22,600	23,200
	Erythrocytes	6,475,000	6,400,000	6,375,000	6,350,000
438	Leucocytes	23,200	24,400	25,000	24,800
	Erythrocytes	6,050,000	6,450,000	6,250,000	5,985,000

TABLE II

Comparison of Counts Immediately after Bleeding and after Storage. Blood Samples Drawn in Rapid Succession from Swine 395

Time of storage	Cells	Heparin (1 mg. per 5 cc. of blood)	Potassium oxalate (2 mg. per cc. of blood)	Sodium citrate (2 mg. per cc. of blood)
		per cu. mm.	*per cu. mm.*	*per cu. mm.*
Within 1 hour following bleeding	Leucocytes	18,800	18,100	17,400
	Erythrocytes	6,562,500	6,487,500	6,225,000
After 7 hours at room temperature (72°F.) and 17 hours in refrigerator	Leucocytes	17,900	19,300	18,050
	Erythrocytes	6,715,000	6,575,000	6,600,000

Blood Changes in Experimentally Induced Hog Cholera

On one or more days preceding inoculation normal counts were obtained on the animals. The virus was administered either intraperitoneally, intracutaneously, subcutaneously or intracorneally,

the mode of administration or amount given (within the range used in these experiments) being immaterial to the results as regards blood cell alterations. Five of the six animals studied were killed when moribund, for pathological material and the other (Swine 432) was allowed to die of the disease.

The results were, in essential detail, the same for the six animals (Table III). All showed a progressive moderate anemia, the attempt at regeneration being demonstrated by showers of nucleated red cells appearing in the circulation rather early in the disease. The leucopenia in all cases was extreme and in five of the six animals was very evident even as early as 48 hours following inoculation. It preceded the temperature reaction or any of the clinical manifestations of the disease. The decrease in leucocytes was rather rapidly progressive so that by the time the temperature reaction was definitely established counts ranging from 2,000 per cu. mm. or lower to 4,000 per cu. mm. were the rule. This extreme leucopenia was maintained, with irregular fluctuations, over a period of from 8 to 13 days, in animals living long enough, when the leucocyte counts rose somewhat to establish a more moderate leucopenia. At no time, however, did the white cell counts reach levels approaching normal. That these variations were genuine and not merely a matter of blood dilution was established by determinations which showed that the corpuscle volume and total solids of the whole blood and plasma were unchanged.

Study of the fixed films indicated that the polymorphonuclear leucocytes were most affected. The leucopenia, however, involved the lymphocytic series as well. Two typical protocols are given in Table IV. Preliminary studies of blood supravitally stained with neutral red and Janus green indicate that differential counts on fixed blood smears do not truly represent the actual hematological picture, for supravitally stained preparations, of the blood which showed no polymorphonuclear neutrophiles and eosinophiles in fixed preparations, frequently yielded these cells very badly damaged, much vacuolated, and apparently very fragile.**

The total leucocyte counts of normal swine blood containing either heparin or potassium oxalate as anticoagulants do not change on

** These examinations were aided by the personal advice and assistance of Dr. Florence R. Sabin and Dr. Charles A. Doan.

TABLE III

Blood Changes in Experimental Hog Cholera

Time after inoculation (days)	Swine 392 Temperature °C	Swine 392 Erythrocytes per cu. mm.	Swine 392 Leucocytes per cu. mm.	Swine 393 Temperature °C	Swine 393 Erythrocytes per cu. mm.	Swine 393 Leucocytes per cu. mm.	Swine 431 Temperature °C	Swine 431 Erythrocytes per cu. mm.	Swine 431 Leucocytes per cu. mm.	Swine 432 Temperature °C	Swine 432 Erythrocytes per cu. mm.	Swine 432 Leucocytes per cu. mm.	Swine 434 Temperature °C	Swine 434 Erythrocytes per cu. mm.	Swine 434 Leucocytes per cu. mm.	Swine 435 Temperature °C	Swine 435 Erythrocytes per cu. mm.	Swine 435 Leucocytes per cu. mm.
Normal	38.8	6,906,000	14,083	38.7	7,175,000	16,040	39.0	6,965,000	16,160	38.8	7,220,000	17,440	38.6	6,560,000	23,700	38.6	6,930,000	19,740
(treatment)	4.5 cc. hog cholera virus intraperitoneally			0.001 cc. hog cholera virus intraperitoneally			0.4 cc. hog cholera virus (serum) intracutaneously			0.1 cc. hog cholera virus (serum) into cornea			0.4 cc. hog cholera virus (serum) intracutaneously			1 cc. hog cholera virus intraperitoneally		
2	39.4	7,155,000	8,550															
3							39.2			39.4								
4	41.5	6,875,000	4,800	40.4	7,165,000	7,740	40.7	6,440,000	8,640	39.9	7,010,000	15,540	38.9	6,385,000	9,600	39.2	6,030,000	11,880
5	40.9	7,460,000	4,340	41.0	7,150,000	8,300	41.5	6,600,000	7,940	39.5		13,440	39.5	6,350,000	6,400	41.3	5,685,000	4,100
6				41.0	5,885,000	6,100	41.5		3,600	39.8		13,900						
7	41.2	6,400,000	2,840				41.6		4,420	39.8		11,440	40.9	6,365,000	4,940	41.0	5,230,000	5,640
8				41.5	5,925,000	4,250	41.5	5,925,000	5,240	41.8	5,400,000							
9	41.2	6,025,000	2,600	41.6	5,925,000	5,050	42.0	6,600,000	4,240	42.0	6,415,000	6,300	41.4	5,830,000	4,340	41.8	6,550,000	6,440
10		5,550,000	4,700				42.1	7,150,000	4,640	41.2	6,375,000	3,840						
11							41.6	6,800,000		42.8		4,000	41.6	5,935,000	1,480			
12										42.2	5,800,000	2,900						
13	41.0	5,110,000		41.0	5,110,000	7,050	41.0		3,200			1,640	41.2	5,875,000	3,410			
14							40.4		3,440	40.5	6,500,000	1,640						3,600
15							40.3		3,540	40.1	7,150,000	2,540	41.1	5,875,000	3,410			
17							39.5	6,100,000	3,800	39.0	6,725,000	8,040	40.0	5,000,000	2,900			
18							39.5	6,050,000	5,900	38.5	6,660,000	9,320		5,650,000	3,010			
19	40.1	4,662,000		40.1	4,662,000	4,450	38.5	7,000,000	8,480	38.6	6,600,000	7,520			3,610	40.0	5,110,000	6,440

20	38.4	5,550,000	8,880	37.0	5,865,000	9,440
21	38.8	6,465,000	12,440	37.0	6,465,000	8,400
22	39.2	5,785,000	9,960	35.8	6,700,000	9,500
23	38.8	5,600,000	8,240	36.8	6,050,000	6,440
24	39.2	4,887,000	11,540	36.2	5,100,000	5,400
25	39.5	4,910,000	11,360	39.5	6,035,000	9,100
26	38.9	5,185,000	11,880	38.9	5,365,000	7,840
28	39.3	3,885,000	10,600			
30	40.0	3,425,000	14,140			
31	39.3	2,310,000	9,600			
32	39.6	2,925,000	6,240			
33	39.5	3,550,000	7,440			
34	40.1	3,075,000	8,300			
35	40.1	3,060,000	10,740			
36	39.5	3,125,000	5,940			
37				40.7	4,575,000	2,320

TABLE IV

Differential Counts in Experimental Hog Cholera

Time after inoculation	Leucocytes	Polymorphonuclear neutrophiles	Polymorphonuclear eosinophiles	Polymorphonuclear basophiles	Lymphocytes	Monocytes	Endothelial cells	Atypical or questionable cells	Nucleated red blood cells	Temperature
days	per cu. mm.	per cu. mm.	per cu. mm.	per cu. mm.	per cu. mm.	per cu. mm.	per cu. mm.	per cu. mm.	per cu. mm.	°C.
Swine 432										
Normal	17,440	5,319	1,482	261	9,680	698	0	0	610	38.8
0.1 cc. hog cholera virus intracorneally										
2	15,540	3,186	1,243	78	10,489	544	0	0	78	39.4
3	13,440	2,486	1,882	67	8,736	269	0	0	67	39.9
4	13,900	2,502	1,460	0	9,173	765	0	0	0	39.5
5	11,440	1,773	572	57	8,466	572	0	126	0	39.8
7	6,300	4,221	63	0	1,386	504	0	0	662	41.8
8	3,840	2,535	192	0	998	115	0	0	499	42.0
9	4,000	1,480	80	0	2,240	200	0	58	300	41.2
11	2,900	928	0	0	1,682	232	0	0	0	42.8
12	1,640	623	0	0	787	230	0	213	0	42.2
14	1,640	246	0	0	919	262	0	0	0	40.5
15	2,540	330	0	0	1,753	432	25	161	241	40.1
17	8,040	1,930	0	0	4,100	1,849	0	186	0	39.0
18	9,320	2,237	0	0	4,288	2,423	186	226	75	38.5
19	7,520	1,579	0	0	3,308	1,805	602	189	283	38.6
21	9,440	2,360	0	0	5,475	1,227	189	84	84	37.0
22	8,400	2,100	0	54	5,880	336	0	0	0	37.0
23	9,500	3,705	0	0	4,560	1,045	190	193	64	35.8
24	6,440	2,640	0	0	2,190	129	1,288	0	54	36.8
25	5,400	1,998	0	0	2,808	108	432	91	91	36.2
26	9,100	3,731	0	0	4,186	1,001	91	0	78	39.5
28	7,840	4,547	0	0	2,430	314	549	0		38.9
Swine 434										
Normal	23,700	4,977	1,659	0	16,590	474	0	0	0	38.6
0.4 cc. hog cholera virus 394 intracutaneously										
2	9,600	960	576	0	7,776	288	0	0	96	38.9
3	6,400	1,600	576	0	3,584	512	0	128	192	39.5
5	4,940	444	296	0	3,806	395	0	99	2,124	40.9
7	4,340	1,606	0	0	2,387	304	0	43	1,042	41.4
9	1,480	281	0	0	1,154	45	0	0	0	41.6
11	3,440	722	0	0	2,374	310	0	34	69	41.2
13	2,900	145	29	0	2,349	377	0	0	58	41.6
14	3,040	0	0	0	2,766	213	0	61	30	41.1
17	3,640		0	23	2,065	162	0	0		40.6
20	2,320	70	0						0	40.7

incubation for periods of 7 hours at 37°C. Blood from early cases of hog cholera, however, when incubated for 7 hours with heparin as anticoagulant, is characterized by a further progress in leucopenia. Potassium oxalate or sodium citrate used as an anticoagulant for similar samples of blood inhibits this progress in the leucopenia. A typical example is given in Table V.

TABLE V

The Effect on the Leucocyte Count of Incubating Normal and Cholera Swine Blood— Swine 441

Time after inoculation	Temperature	Heparin (1 mg. per 5 cc. of blood as anticoagulant)			Potassium oxalate (2 mg. per cc. of blood as anticoagulant)			Sodium citrate (2 mg. per cc. of blood as anticoagulant)	
		Incubated			Incubated			Incubated	
		0 hrs.	7 hrs.	24 hrs.	0 hrs.	7 hrs.	24 hrs.	0 hrs.	7 hrs.
days	°C.	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*
Normal	37.8	26,600	24,160		25,700	27,200			
Inoculated intraperitoneally with 1 cc. of hog cholera virus									
1	37.7	14,040	11,940		15,540	14,200			
2	38.7	9,900	8,700		9,240	9,940			
3	38.5	10,740	7,100		10,100	9,200			
4	40.5	6,940	7,240*		5,860	6,700*			
5	40.6	7,500	5,440	5,140	6,480	6,960	6,400		
6	40.6	4,600	2,800		5,040	5,000		5,300	4,500

* Many small, round forms.

Blood Changes in Field Cases of Hog Cholera

It seemed possible that factors other than those encountered in experimentally induced hog cholera might cause the blood changes in field cases of the disease to be less characteristic. Consequently, data were obtained on animals sick with acute hog cholera from five widely separated herds. These data are presented in Table VI. The leucopenia is just as characteristic in naturally occurring hog cholera as it is in that experimentally induced, and the leucocyte counts on normal hogs under field conditions compare very well with those recorded for our normal laboratory animals. They range from

14,000 per cu. mm. to 26,500 per cu. mm. and the average of fifteen such animals was 21,800 leucocytes per cu. mm.

The Leucopenia of Hog Cholera as a Diagnostic Aid

At present, certainty in the diagnosis of hog cholera is to be secured only by the reinoculation of filtered materials into healthy animals. It would seem, therefore, that any additional clinical evidence which should be precise and readily obtained would be both welcome and useful, even though it might lack the quality of absolute certainty.

TABLE VI

Counts on Field Cases of Hog Cholera

Case No.		Erythrocytes	Leucocytes	Remarks
		per cu. mm.	*per cu. mm.*	
Woodbury	1	3,850,000	960	"Vaccination break"
"	2 A	5,575,000	6,520	" "
"	3	5,400,000	9,550	" "
"	2 B	4,925,000	9,400	" "
"	13	4,815,000	6,140	" "
Trenton	1		7,040	Spontaneous
"	2		1,200	"
Plainview	1		4,740	"Vaccination break"
Whittier	2		5,000	" "
"	3		4,840	" "
Kalona	1		7,800	Spontaneous
"	2		6,140	"
"	3		7,300	"
"	4		2,900	"

The leucopenia observed in the disease is so extreme that it has seemed possible to use it as a discriminating aid in the diagnosis of the disease. With this in mind, further work has been done with swine diseases, often clinically confused with cholera, and with phases of hog cholera itself, that might conceivably show a different blood picture.

Following immunization of swine against hog cholera, using the so-called simultaneous or double treatment, vaccination breaks not infrequently occur; that is, during a period ranging from a few days to two weeks or longer after immunization a few animals in the herd or the whole herd become sick. Under such conditions it is of the

utmost importance to both the owner of the herd and the veterinarian in charge to determine promptly whether the disease is hog cholera or something else.   For this reason we have determined the effect on the blood picture of the administration to normal swine of hog cholera virus and antiserum, in the quantities used in conferring active immunity against hog cholera.   Dr. J. T. McGrann of Trenton, New Jersey, kindly made this material available to us.   Leucocyte counts were made on animals just before the immunizing injections and at the end of 1, 3, and 5 days.   The data obtained in this experiment are given in Table VII.

A condition very often suspected and very seldom seen in swine is hemorrhagic septicemia.   We have tried for a long while, unsuccess-

TABLE VII

The Effect of Anti-Hog Cholera Immunization on the Leucocyte Count of Swine

Time after immunization	Swine 401 Leucocytes	Swine 403 Leucocytes	Swine 404 Leucocytes	Swine 405 Leucocytes
	per cu. mm.	*per cu. mm.*	*per cu. mm.*	*per cu. mm.*
Normal	14,040	25,440	25,200	26,440
Double treated with Pitman-Moore hog cholera virus and serum				
1 day	28,840	23,880	30,900	39,800
3 days	18,140	25,340	37,100	38,100
5 days	17,740	22,540	27,140	34,540

fully, to obtain an uncomplicated case.   Failing that we have resorted to a study of swine inoculated with *Bacillus suisepticus*.   A protocol of the experiment is given in Table VIII (Swine 394 and 398).

It was conceivable that secondary invasion of an animal, suffering from hog cholera, by an organism normally capable of producing a leucocytosis would serve to obscure the characteristic cholera leucopenia and make differential diagnosis by the leucocyte count impossible under such conditions.   To test this possibility two hogs were inoculated with hog cholera virus and the disease allowed to progress for 7 days until the animals were well into the stage of leucopenia. They were then inoculated with *B. suisepticus* in a dosage that was known, by reason of the experiment just cited, to be capable of producing a marked leucocytosis in normal swine.   Blood counts were

done at 2, 6 1/2, and 11 1/2 hours after inoculation and daily for 3 days more.   At no time did a leucocytosis result (Table VIII, Swine

TABLE VIII

The Effect on the Blood Picture of Inoculating Normal and Cholera Infected Swine with B. suisepticus

Time after inoculation		Swine 396			Swine 397		
		Tempera-ture	Erythrocytes	Leucocytes	Tempera-ture	Erythrocytes	Leucocytes
days	*hrs.*	°C.	*per cu. mm.*	*per cu. mm*	°C.	*per cu. mm.*	*per cu. mm.*
Normal		39.5	6,100,000	17,350	38.9	7,037,000	17,050
0.1 cc. hog cholera virus intraperitoneally							
1		39.4	6,100,000	11,600	38.6	6,920,000	17,224
2		40.2	6,512,000	8,290	39.4	6,740,000	8,100
3		41.0	5,837,000	6,250			
4					41.0	7,230,000	5,900
7		42.0	6,412,500	8,250	41.5	7,300,000	5,280
5 cc. of a 24 hour broth culture of *B. suisepticus* (376D) subcutaneously							
	2	42.2	6,325,000	5,800	42.0	7,400,000	1,050
	6½	41.6	6,462,000	9,400		6,712,000	3,850
	11½		6,250,000	7,060			
8		41.1	6,637,000	5,300	42.0	6,540,000	6,100
9		42.0	5,150,000	5,150	41.0	5,800,000	3,600
10		41.8	5,425,000	9,400			
		Swine 394			Swine 398		
Normal		39.2	7,112,000	14,650	38.6	7,090,000	21,300
5 cc. of a 24 hour broth culture of *B. suisepticus* (376D) subcutaneously							
	2		7,137,000	19,300	39.5	7,260,000	21,660
	6½	41.6	6,900,000	32,300	41.0	7,075,000	41,750
	11½	41.4	6,700,000	27,300			
1		39.5	6,650,000	26,200	39.6	5,890,000	40,500
2		39.0	6,412,000	15,100	39.0	6,020,000	20,900
3		39.0	6,050,000	14,600			

396 and 397).   In fact, the immediate reaction was to produce a more profound leucopenia.   Inoculation of cholera-sick swine with hog cholera bacilli resulted in a similar reaction.

Infectious enteritis, a disease very prevalent in swine in the middle western states in the summer and fall, may in its early stages be confusing diagnostically. Data concerning the leucocyte reaction to this disease were obtained on field cases (Table IX) and experimentally

TABLE IX

Leucocyte Counts on Field Cases of Infectious Enteritis

Case No.	Leucocytes	Case No.	Leucocytes
	per cu. mm.		*per cu. mm.*
North Liberty 1	25,700	North Liberty 5	16,240
" " 2	33,640	Kalona 1	33,540
" " 3	18,900	" 2	37,500
Sharon 1	20,840	Tiffin 1	32;300
Watkins 1	16,900	" 2	31,600
" 2	33,140	" 3	32,320
Plainview 2	44,500	" 4	34,000
North Liberty 4	56,460		

TABLE X

Leucocytes per Cubic Millimeter of Blood in Cases of Experimentally Induced Infectious Enteritis

Time after infection	Swine 8232	Swine 8233	Swine 8234	Swine 8236	Swine 8238	Swine 8239
Normal	26,600	34,940	22,040	18,800	26,540	22,800
Fed 200 cc. of a 48 hour broth culture of *B. suipestifer*						
12 hrs.	27,600	34,240	20,340	29,800	26,300	20,440
24 "				33,400	34,100	18,540
36 "	29,600	22,200	18,200			
2 days				32,140	26,340	8,740
3 "	44,300	31,000	16,660	20,800	40,540	18,800
4 "	34,240	36,400	21,300	25,400		37,400
5 "	34,140		35,400	24,100		29,700
6 "	38,900		30,500	20,740		17,400
7 "	37,100			16,600		

induced cases (Table X). In the field cases the predominant reaction was a slight to moderate leucocytosis and in no instance was leucopenia observed. The greatest relative increase was in the polymorphonuclear leucocytes. These findings are confirmatory of earlier unpub-

lished observations by Dr. H. E. Biester. Through the kindness of the latter and of Dr. Charles Murray, Iowa State College, Ames, we were enabled to observe variations in the leucocyte counts of animals in which infectious enteritis had been experimentally induced. The

TABLE XI

Counts in Field Cases of Swine Influenza

Case No.	Approximate day of disease	Leucocytes** per cu. mm. of blood	Erythrocytes per cu. mm. of blood
West Branch 1	3rd	16,740	
" " 2	5th	23,900	
Windham 1	3rd	10,100	
" 2	4th	19,600	
" 3	3rd	16,100	
" 4	4th	16,640	
" 5	4th	25,240	
Wellman 1	5th	11,100	
" 2	5th	13,500	
" 3	5th	8,820	
Iowa City 4	3rd	14,900	
" " 5	3rd	13,600	
" " 6	3rd	23,600	
North Liberty 1	3rd	11,200	6,375,000
" " 2	3rd	16,040	7,750,000
" " 3	3rd	12,800	7,150,000
Lone Tree 1	2nd	16,800	
" " 2	2nd	19,700	
" " 3	5th	22,100	
" " 4	3rd	13,140	
North Liberty 4	5th	22,540	
" " 5	3rd	9,200	
Mount Vernon 1	2nd	10,000	
" " 2	2nd	10,300	
" " 3	3rd	19,000	

** Leucocyte counts done on the blood of 24 normal hogs during the late fall and winter months averaged 24,600 leucocytes per cu. mm. of blood, with extremes of this series at 19,000 and 35,000 leucocytes per cu. mm.

findings agreed in essential detail with those on field cases of the condition, in that there was a slight to moderate leucocytosis in all cases. One animal, Swine 8239, developed a leucopenia on the 2nd day following infection that was in the upper range of those observed

for cholera. However, the change was very transient and was followed by a rather marked leucocytosis on the 4th day.

Swine influenza (hog flu) is very prevalent among swine in the middle western states during the fall and winter months and at times is very difficult to differentiate from hog cholera. It occurs at a time of the year when, even on the farms where hog cholera immunization is regularly practiced, large numbers of the fall pigs have not yet been vaccinated. While swine influenza occurring in hogs that are cholera immune is readily diagnosed as such, in animals that are not cholera immune a definite differentiation has not been possible at times until the disease has progressed for several days. Data concerning the leucocyte reaction in swine influenza were obtained in Iowa in the fall of 1928. Counts were made on both cholera-immune and non-immune animals suffering from swine influenza (Table XI). The predominant reaction was a slight to moderate leucopenia. The greatest relative decrease was in the polymorphonuclear leucocytes. The degree of leucopenia attained was much less than that occurring in hog cholera and in only two out of twenty-five cases of swine influenza were the counts low enough to have been possibly confused with the leucopenia of hog cholera. The leucopenia in these two animals reached the upper limits of the hog cholera leucopenia but the condition was readily differentiated when leucocyte counts on other sick animals in the same herds were conducted. The data presented here would indicate that the two conditions, hog cholera and swine influenza, could be differentiated as herd infections by consideration of the leucocytic reaction.

A few leucocyte counts on field cases of swine, heavily infested with ascaris, and on animals suffering from an obscure type of posterior paralysis, showed that in neither of these conditions was a leucopenia developed, a slight to moderate leucocytosis being the rule.

DISCUSSION

In blood kept from coagulating by small amounts of heparin the leucopenia of hog cholera can be made to progress still further *in vitro* by incubating. The decrease in cells *in vitro* might conceivably be due to the presence of a lytic substance active against leucocytes. To test this possibility samples of normal swine blood to which blood

serum from cholera-sick swine had been added were incubated for varying periods of time and then counted. No decrease in the number of leucocytes could be demonstrated. That the white blood cells are fragile in hog cholera is indicated by the supravital observations. It seems likely that the decrease observed *in vitro* on incubation is due to the fragmentation of these fragile cells. Potassium oxalate and sodium citrate in some way inhibit the fragmentation. Whether the damage to the leucocytes is due to the virus itself or to some toxic product capable of action only *in vivo* has not as yet been determined.

So far as we have gone, severe leucopenia has been found to be present in only one disease of swine that is clinically confusing with hog cholera, namely swine influenza, and then only occasionally. It is believed by us that a leucocyte count of 8,000 per cu. mm. or less on three sick animals in a suspected herd can be relied on as indicating that the condition is hog cholera.

SUMMARY AND CONCLUSIONS

1. Prolonged and systematic examination of blood from swine with hog cholera has failed to reveal any formed element that could be identified with the etiological virus. Culture has likewise been unsuccessful.

2. The quantitative blood changes in hog cholera consist in a slowly progressive anemia, usually moderate in degree, and a rapidly progressive severe leucopenia affecting cells of the polymorphonuclear series most markedly but also including those of the lymphocytic series.

3. Incubation of hog cholera blood results in a further progress of the leucopenia, *in vitro*, if heparin has been used as the anticoagulant, but there is no significant change if potassium oxalate or sodium citrate has been used.

4. Consideration of the leucocytic reactions prevailing in experimental infection with *B. suisepticus*, in infectious enteritis, in swine influenza, following successful immunization against hog cholera, and following infection of cholera-sick swine with secondary invaders indicates that the leucocyte count would be of aid in the differential diagnosis of hog cholera.

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THE ELECTRICAL CHARGE OF BACTERIOPHAGE

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Early experiments on the character of the electric charge carried by the Bacteriophage corpuscle yielded entirely conflicting results. Thus, v. Angerer (1) found that lytic particles suspended in an electric field migrated toward the anode while Koch (2) employing similar methods obtained migration in the opposite direction. Indirect determinations of the charge by means of adsorption experiments likewise failed to furnish conclusive evidence. For example, Gildemeister and Herzberg (3), and Kramer (4) agreed that Bacteriophage is adsorbed only by positive colloids. Fränkel and Schultz (5) and Prausnitz and Firle (6) found that adsorption occurs with negative colloids only, whereas Marcuse (7) and Seiffert (8) obtained adsorption with positive, negative and amphoteric colloids. It seems likely, as Bronfenbrenner (9) has pointed out, that much of this divergence of opinion has arisen from failure to adequately control the experimental conditions.

Recent careful work by Todd (10), utilizing a cataphoresis cell of his own design, has clearly proven that the Bacteriophage corpuscle bears a negative charge over a range of H-ion concentrations from pH. 3.6 to pH. 7.6. We were interested in repeating his experiments and in extending the observations to determine whether or not the charge might be reversed upon further lowering the pH.

Apparatus

Type A.: Since Todd's convenient apparatus was not available and we were unable to construct the cell because of the hollow-barreled stopcock required, one of us (A.P.K.) designed the cell shown in Figure 1.

Moist glass wool or filter paper shreds are tamped lightly into a layer 0.5 cm. thick above the closed side-arm stopcocks. The latter are opened, the limbs of the apparatus fitted with cotton plugs, and sterilization is accomplished by

autoclaving. After the apparatus is cool, both side stopcocks being open and the central limb closed off, appropriate buffer solution is poured in until the side arms are filled to the level indicated in Figure 1. The buffer solution consists of just sufficient buffer mixture added to water so that the resulting Bacteriophage suspension will be of desired pH.
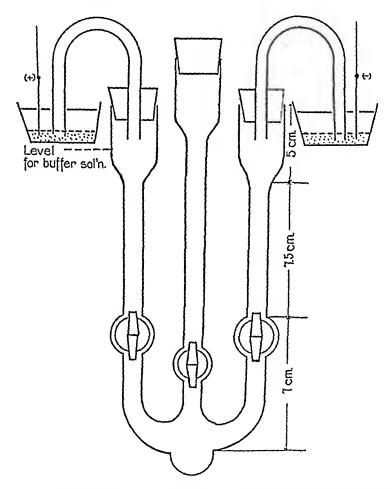
FIGURE 1. Cataphoresis cell. Type A. Anode: silver foil dipping into salt crystals. Water to level of upper line. Cathode: copper wire dipping into crystals of cupric chloride. Water to level of upper line.

The cotton plugs in the side arms are now replaced by single hole stoppers carrying glass U-tubes filled with a 3% agar gel containing 1% C. P. sodium chloride. The stoppers and bridges are conveniently made up in quantity beforehand. We usually sterilize 15 such units in a jar and fill the inverted U-tubes with sterile agar-salt mixture just before using. The stoppers should have cut in them a narrow groove parallel to the U-tube to allow for escape of air.

Bacteriophage* is placed in the central limb to a level above that of the buffer solution in the side arms and after a few moments is carefully allowed to pass into the mixing chamber.   The bulb is spherical so that the fine stream of Bacteriophage entering it tends to produce currents whch move circularly and therefore cause no diffusion of Bacteriophage up the side arms.   When sufficient lysate has been run in (ordinarily 2–3 cc.) the central stopcock is closed and a direct current of 60–200 volts and 5–12 milliamperes passed for several hours.   Samples are then taken with sterile pipettes from each side arm and from the bulb and are tested in the usual manner against known susceptible organisms for the presence of Bacteriophage.   It is advisable to ascertain the pH. of anode, cathode, and mixing bulb samples after each run.   The bulb sample may be obtained by passing a fine capillary pipette through the open central stopcock and aspirating a small amount.

Type B: The apparatus is explained in the accompanying diagram and requires no special mention except to note that convenient dimensions for the tubes are 15 cm. by 2.5 cm.   The assembled glass parts are autoclaved and the bridges filled with sterile 3% agar containing 1.0% C. P. sodium chloride.   This is readily accomplished by pipetting the agar into the inverted upper bridges and by resting the distal ends of the lower bridges against a flat surface during the filling process.   After the agar is hard the lower bridges and stoppers are fitted in place; 20 cc. of purified 0.5% agar† containing 0.2% C. P. sodium chloride is poured into each cylinder, observing sterile precautions.   When the agar is hardened, 30 cc. of Bacteriophage suspension adjusted to the pH. at which the charge is to be determined, is layered over it and the upper stoppers placed in position.

As will be seen from the diagram, the anode is at the bottom in one set-up and at the top in the other.   A D. C. current of 100–200 volts and from 5–12 milliamperes is passed through the cylinders for from 4–10 hours.   At the end of this time the broth is taken up by pipette, discarded, and the upper surfaces of the agar washed with several portions of sterile saline solution.   The lower bridges and stoppers are removed and the agar allowed to slide out gradually.   Each agar plug is sliced into thin sections with a sterile spatula as it comes out.   These are received in a sterile Petri dish, discarding the layer 0.5 cm. thick nearest the broth. The remainder is thoroughly macerated for two hours with 10–20 cc. of sterile water.   The agar particles are permitted to settle and the supernatant fluid is then tested in the usual manner, employing both solid and liquid media, for the presence of Bacteriophage.

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*We have worked entirely with highly virulent anti-coli Bacteriophages, (several separate races) some of which were active against dysentery organisms. In the experiments with Type A apparatus the lysates were always previously purified and concentrated by the method of Krueger and Tamada (11) to remove as much extraneous matter as possible.

† This "purified" agar is prepared according to the method of Dominikiewicz (12).
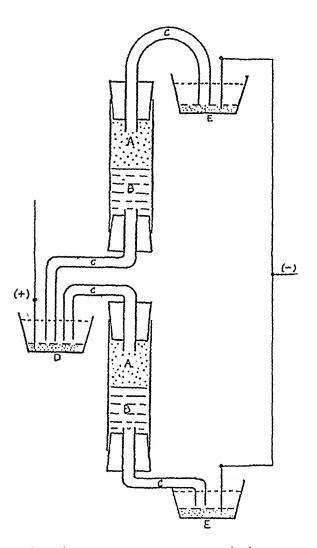
FIGURE 2. Cataphoresis cell.   Type B.   *A*-Bacteriophage suspension.   *B*-20 cc. purified 0.5% agar containing 0.2% C. P. sodium chloride.   *C*-Agar bridges filled with 3% agar containing 1.0% C. P. sodium chloride.   *D*-Anode.   Silver foil dipping into sodium chloride crystals.   Water to level of upper line.   *E*-Cathodes.   Copper wire dipping into crystals of cupric chloride.   Water to level of upper line.

EXPERIMENTAL RESULTS

With the Type A apparatus the results shown in Table I were obtained, each experiment being run in duplicate.

Attempts to carry on similar experiments below pH. 3.5 consistently

TABLE I

Summary of Cataphoresis Experiments with Type A Apparatus

pH.	Buffer	Time	Volts	Milli-amperes	Bacteriophage Present in		
					Anode Sample	Cathode Sample	Bulb Sample
		hrs.					
9.0	Borate-HCl	6.0	204	12.2	+	—	+
8.5	" "	4.0	140	5.3	+	—	+
8.0	" "	4.0	145	6.0	+	—	+
7.5	KH$_2$PO$_4$-NaOH	5.0	148	6.2	+	—	+
7.0	" "	4.0	140	8.0	+	—	+
6.5	" "	4.0	132	10.0	+	—	+
6.0	" "	6.0	130	10.2	+	—	+
5.5	KH Phthalate-NaOH	4.0	141	10.0	+	—	+
5.0	" "	5.0	132	11.0	+	—	+
4.5	" "	5.0	140	5.6	+	—	+
4.0	" "	4.0	136	8.0	+	—	+
3.6	KH Phthalate-HCl	5.0	148	7.2	+	—	+
3.5	" "	5.0	99	6.5	—	—	—
3.5	" "	3.0	110	8.0	+	—	+
3.5	HCl	4.0	90	7.2	—	—	—
3.5	KH Phthalate-HCl	2.0	109	8.2	+	—	+
3.4	" "	2.0	113	6.8	—	—	—
3.4	" "	1.0	115	8.0	—	—	—

TABLE II

Summary of Cataphoresis Experiments with Type B Apparatus

pH.	Buffer	Time	Volts	Milli-amperes	Bacteriophage Present in		
					Anode Agar	Cathode Agar	Supernatant fluid at end of run
		hrs.					
3.60	KH Phthalate-HCl	4.0	168	9.2	+	—	+
3.50	" "	5.0	99	6.5	+	—	—
3.40	" "	5.5	100	8.8	+	—	—
3.35	" "	5.5	89	11.0	—	+	—
3.30	" "	4.0	95	12.0	—	+	—
3.25	" "	7.0	100	8.0	—	+	—
3.20	" "	3.5	125	5.0	—	+	—
3.15	" "	7.0	100	9.6	—	+	—
3.10	" "	5.5	102	9.2	—	+	—

met with failure due to inactivation of the Bacteriophage by the relatively high concentration of H-ions to which the lytic agent was exposed. It was this difficulty that led us to adopt the type B apparatus. Data from experiments with the latter type of cell are summarized in the appended table. Many more experiments with other races of anti-coli Bacteriophage have given like results (Table II).

<div align="center">DISCUSSION</div>

The data given in Table I confirm the results reported by Todd; namely, that Bacteriophage possesses a negative charge within the range from pH. 3.6 to pH. 7.6. Our experiments further indicate that the charge is a negative one from pH. 3.5 to pH. 9.0. It would seem from Table II that as one adjusts the pH. of a Bacteriophage suspension below pH. 3.4 there occurs a reversal of the electrical charge carried by the lytic particles or the aggregates to which they adhere.

As has been noted, it is not possible to elicit this reversal phenomenon if one employs a cataphoresis cell in which the Bacteriophage is exposed to high H-ion concentrations for any length of time, because of inactivation. In the Type B apparatus the original lysate is of the requisite low pH. for acquisition of a positive charge but the corpuscles rapidly migrate from this environment into the agar gel and therefore are not inactivated. The agar has a pH. of 5.1–5.3 and one naturally wonders why the positively charged lytic particles traversing this medium do not have their charges neutralized or reversed. We are unable to explain this apparent paradox. Furthermore, it seems fair to assume that the Bacteriophage travels through the more fluid portions of the gel in the interstices between the granules, and in these areas is exposed to electroendosmotic currents. However, such flows do not appear to play a major rôle in determining the direction of migration under the experimental conditions employed, for experiments conducted with the same type of cell over a range from pH. 9.0 to pH. 3.40 always resulted in a migration of Bacteriophage to the anode. This would hardly have been the case were there constantly a strong electroendosmotic flow toward the cathode.

Whether we are dealing with the charge borne by the actual lytic substance itself is another question. Recent work by Krueger and Tamada (13) indicates that relatively pure Bacteriophage prepared by

diffusion of the corpuscles through agar has a smaller particle size than the Bacteriophage as it occurs in ordinary broth suspensions, and that such "pure" Bacteriophage tends to form larger aggregates when placed in contact with proteins or protein derivatives. There is no proof however that the smaller particles consist of the lytic principle only and it is quite possible that the charge we are determining is that of a micellar aggregate which carries with it adsorbed Bacteriophage.

CONCLUSIONS

1. Two types of cataphoresis apparatus for determining, under aseptic conditions, the charge carried by biologically active substances, such as Bacteriophage are described. One cell depends upon the electrophoresis of particles into agar and their subsequent re-suspension in a fluid medium for testing purposes. This cell has certain advantages when employed in connection with agents of small dimensions ordinarily inactivated by prolonged exposure to required test conditions.

2. Several separate races of anti-coli Bacteriophage have been found to bear a negative charge within a range of H-ion concentrations from pH. 9.0 to pH. 3.4. At pH. 3.35 and below, the lytic particles migrate through agar to the cathode. It is likely that the reversal in direction of migration is due to the assumption of a positive charge by the Bacteriophage corpuscle.

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THE FATE OF TUBERCLE BACILLI IN THE ORGANS OF REINFECTED RABBITS

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In a recent review of our present knowledge of immunity in tuberculosis Löwenstein (1) says, "What is the fate of the newly introduced tubercle bacilli in the tuberculous organism? Easy as it would seem to decide this question, yet how little do we now know about it with certainty." It is generally assumed that tubercle bacilli of reinfection are destroyed (2), although Krause and Willis (3) are of the opinion that immunity to reinfection is primarily due not to specific bacteriolysins, which destroy the bacilli, but to the interposition of an inflammatory barrier about them, which checks their spread in the body. There have been many experiments purporting to demonstrate a lysis of tubercle bacilli in the previously infected animal (4). These conclusions however have usually been based upon the reduction in numbers of stainable bacilli of reinfection and the tinctorial changes of those found. But when animal inoculation (5) has been used to determine the fate of tubercle bacilli of reinfection they have been found to persist in a virulent form.

In a previous study (6) cultural methods were used to determine the fate of both human and bovine tubercle bacilli in the organs of intravenously infected normal rabbits. It was shown that at first both the human and the bovine tubercle bacilli multiply in all the organs. The human tubercle bacillus grows faster at first than the bovine bacillus, but soon a change takes place, which causes its destruction, first in the liver, spleen, and bone marrow, and later in the lung and kidney. The bovine tubercle bacillus multiplies in these organs more slowly at first, but here too destruction takes place in the liver, spleen and bone marrow, though later than with the human bacillus, whereas in the lung and kidney multiplication continues without effective opposition until the death of the animal. It was also

shown that the time at which destruction of either type begins and the completeness of destruction depend upon the quantity of bacilli inoculated. The larger the number of bacilli inoculated the more rapid and the more complete will this destruction be, as could be seen in certain organs and with certain dosages.

Thus it would appear that as a result of the growth of both types of bacilli in the rabbit some change takes place in the body, which destroys the bacilli. One would expect that after such a change has once been brought about in the rabbit the introduction of fresh tubercle bacilli from without would be followed by immediate destruction without any preliminary growth. To test this hypothesis, and to ascertain the fate of tubercle bacilli in the organs of reinfected rabbits, the following experiments were performed.

Method

A series of rabbits was inoculated intravenously with 0.001 mg. of a human strain of tubercle bacilli, P-48A, per kilo of body weight. It was shown in the previous study that six months after such an infection the tubercle bacilli had practically disappeared from the liver, spleen, bone-marrow and kidney and had been greatly reduced in number in the lung. At about this time, therefore, these rabbits were given intravenously a reinfecting dose of 0.01 mg. of the same strain of human tubercle bacilli. Another series of rabbits was similarly infected with the human strain and reinfected with a bovine strain, Bovine C, in the same quantities and by the same route. At the same time two series of normal rabbits were injected intravenously with 0.01 mg. of the same strains used for reinfection, one with the human, and one with the bovine bacilli.

At intervals of 1 day, 1, 2, 4 and 6 weeks and 2 months, equal amounts by weight of suspensions of ground lung, liver, spleen, kidney and bone marrow in varying dilutions were seeded upon the surface of Dorset's and Petroff's media, both directly and after sodium hydroxide treatment. Care was taken to avoid tissue obviously affected by primary infection. At least 3 tubes of each medium were seeded with a given dilution of a given organ, both directly and after treatment. The results obtained were therefore based upon the readings of at least 12 separate tubes seeded with the same quantity of the same tissue or, when 2 dilutions were used, upon 24 tubes, except, of course, when contamination had occurred in some. The method has been described more fully in the previous publication; in the experiments described here larger tubes were used to afford a larger surface area and more accurate enumeration of colonies, and, for the same reason, instead of a strip of celluloid, a strip of spring metal, into which windows of accurately measured square areas had been cut, was used for estimating the number of colonies when these exceeded 200.

The number of colonies of tubercle bacilli appearing upon each tube was repeatedly determined, the final readings being made after 3 months' incubation.

It was shown in the previous study that there is no essential difference between the number of colonies derived from a given dilution of a given organ on either Dorset's or Petroff's media. Therefore for brevity's sake only the highest average figure obtained from the one or the other medium after direct seeding is tabulated. Where all the tubes of direct seeding were contaminated the number of colonies obtained after sodium-hydroxide treatment is given multiplied by 10, for it has been found that the number of colonies as a result of the manipulation in treatment is usually reduced ten times. However only occasionally have these adjusted figures been necessary.

The Fate of Human Tubercle Bacilli of Reinfection in the Organs of Rabbits

In Table I the fate of human tubercle bacilli in the organs of normal and of reinfected rabbits with the resulting pathological changes are compared. The number of colonies derived from the same quantity of a given organ of an infected and of a reinfected rabbit are placed in two columns in juxtaposition. As a rule 3 reinfected rabbits are compared with 2 infected animals for each interval.

In the *lungs* of the infected rabbits the human tubercle bacilli multiply at once, increasing in number continuously to about the 4th week, when the largest number, namely 12,000, was derived from 10 milligrams of tissue; thereafter there is a tendency for a diminution in numbers, though very considerable quantities of tubercle bacilli persist even after 2 months. In the lungs of the reinfected rabbits, on the other hand, neither growth nor destruction is discernible as a definite, continuous tendency, but tubercle bacilli are isolated in extremely variable numbers. These are ascribable to the residual lesions of the primary infection and not to the reinfection, for the normal rabbits primarily infected with 0.01 mg. of tubercle bacilli showed no macroscopic changes in any organ until the fourth week, whereas 7 out of the 8 reinfected rabbits killed within the first 2 weeks of reinfection showed considerable, and at times extensive, tuberculous lesions even 24 hours after reinfection. These lesions are obviously due to the primary infection and not to the reinfection, and the tubercle bacilli isolated in very variable numbers from the lungs of reinfected rabbits are therefore probably residual organisms from the primary infection. Moreover at each interval after reinfection there is at least one rabbit in which the number of tubercle bacilli in the lungs is extremely few. Thus rabbit 15, 24 hours after reinfection, showed 56 colonies; rabbit 17, after a week, 6 colonies; rabbit 20, after 2 weeks, showed 6; rabbit 22, after 4 weeks, showed 10; rabbit 27 showed 75 colonies 6 weeks after reinfection, and none could be isolated from rabbit 28 even 2 months after reinfection. On

TABLE I

Number of Colonies and the Pathological Changes Found in Rabbits Infected, and in Rabbits Reinfected† with 0.01 Mg. B. tuberculosis, Human Type

Interval after infection or reinfection	Rabbit No.		Lung		Liver		Spleen		Kidney		Bone marrow		Pathological findings	
	Reinf.	Inf.	Inf.	Reinf.	Inf.	Reinf.	Inf.	Reinf.	Inf.	Reinf.	Inf.	Reinf.	Infected	Reinfected
1 day		1/14	9	720	14	3*	18	6	0	2	2.5	0.3	Neg.	Num. discrete pulm. tubercles; 1 cortical lesion in left kidney
		2/15	30*	56*	18	12	13	42	0	15	1.4		Neg.	2 isolated pulm. tubercles
		16		1,030		3*		0*		0	2			Small no. of discrete pulm. tuberc.; fibrocaseous tuberc. in cortex and medulla of left kidney
1 week		3/17	48	6	8	0*	40	2	2	0	1		Neg.	Single pulm. tubercle
		4/18	50	61	1	0.3	76	142	0	1	1.5		Neg.	Localized caseous cavities in bases of both lungs; few tubercles at margins; no. of tuberc. in spleen
2 weeks	560	19	40		8		11		3	17	4		Neg.	Neg.; enlarged spleen
	1,580	5/20	123	6*/110	1	243	1	2	176	21	0		Neg.	Isolated margial pulm. tuberc.; isolated cortical tuberc. in left kidney
		6/21		83	8	1,380	10		0*	176/99	1		Enlarged spleen	Several large localized lesions in both lungs, one of these enclosing a cavity
4 weeks	600	7/22	10	60	0.3	596	0	10*	0*	72	0		Num. miliary tubercles with punctate caseation of lung; few to mod. no. of tuberc. in liver, spleen, kidney and bone marrow	2 or 3 isolated tuberc. at anterior margins of both lungs

											Lesions (conglomerate / miliary)	Lesions (discrete)
6 weeks												
8|23	12,500*	3,220	7	4	416	40	2,200	4	0*	0*	Num. miliary conglom. tuberc. in both lungs with caseous centers; occasional tuberc. in spleen; mod. no. of tuberc. in kidney	Numerous discrete pulm. tubercles; few cortical tuberc. in kidneys
24		20		1.5						0		2 or 3 isolated tubercles in lungs; large cortical tuberc. and caseous pus in pelvis of right kidney
9|25	1,820*	4,530*	4	0	105	0	76	22	10	0	Extensive miliary, caseous pulm. tuberc.; slight to mod. miliary tbc. of liver, spleen, kidney and bone marrow	Moderate no. of discrete nodular tubercles and pus containing cavities in both lungs
10|26	1,370	22,070	1	2	0	0.3	10	0	0.6		Moderate no. of tubercles in both lungs; 2 or 3 tubercles in cortex of kidneys	Localized ulcerative pulm. tbc.; 2 cortical tubercles in left kidney
27**	75		0	0	0.3	0	0	3	0			Isolated fibrous pulm. tuberc.; isolated cortical tbc. of both kidneys
2 mos.												
11|28	2,970	0*	1.5	0.3	9	0.3	170	120	0	0	Ext. conglom. miliary tbc. of lungs with multiple foci of caseation; mod. no. of cort. tuberc. in kidneys	2 isolated pulm. lesions, one of these an encapsulated cavity; sev. cort. fibr. lesions in kidney
12	3,800*		0	0	0		1,170	0	0		Ext. conglom. discrete miliary tbc. of lungs with caseous foci. Few cortical tuberc. in kidneys	
13	5,260		0	0	0.3		0	0	0		Numerous discrete tuberc. 2 to 4 mm. with caseation; single cortical tubercle in each kidney	

† The reinfected rabbits had a primary inoculation of 0.001 mg. B. tuberculosis, human type, about six months before reinfection.

* Adjusted from figures obtained after treatment.

** No direct cultures made; figures for this rabbit adjusted from those obtained after treatment.

the other hand the lowest numbers of colonies found in the primarily infected rabbits were 9, 48, 560, 600, 1370 and 2970 for the corresponding intervals. There is thus no clear direct evidence for the multiplication of human tubercle bacilli in the lungs of reinfected rabbits and similarly no evidence of any tuberculous changes in the lungs due to the reinfection.

In the *liver* of normal rabbits the human tubercle bacilli multiply somewhat more slowly than in the lung and reach their height by the second week. Their destruction is rapid from the 4th week, so that 6 weeks after infection they have practically disappeared. In the liver of reinfected rabbits fewer tubercle bacilli are recovered even 24 hours after reinfection than after primary infection. From then on only a few isolated colonies are found. It is noteworthy that although the bacilli of reinfection are destroyed immediately upon introduction without any preliminary growth this destruction is not complete; even 2 months after reinfection some few isolated organisms persist.

In the *spleen* of normal animals multiplication is more rapid than in the liver; this also reaches its height by the second week. Destruction begins about the fourth week; by the sixth week it is well under way and at the end of the second month the bacilli have practically disappeared. Again, as in the liver, the human tubercle bacilli of reinfection are destroyed at once without any preliminary multiplication. The 142 colonies recovered from the spleen of rabbit 18 are obviously due to tubercles found in the spleen and therefore, obviously, are derived from the primary infection, as no macroscopic tubercles are formed in the spleen within a week after infection, when this rabbit was killed.

In the *kidneys* of normal rabbits the bacilli accumulate very slowly; the highest number was obtained in the fourth week; there is a tendency to a slow decline thereafter, but numerous tubercle bacilli persist, in the kidney as in the lung, even 2 months after infection. In the kidney of reinfected rabbits the same result was obtained as in the lung. In the great majority of cases no evidence of growth of the bacilli of reinfection occurred in this organ, although occasionally considerable numbers of tubercle bacilli were found, probably due to the residual lesions of the primary infection.

In the *bone marrow* of normal rabbits multiplication is rapid at first, reaches its height by the second week and then rapidly declines, so that 6 weeks after infection the bacilli have practically disappeared. In the bone marrow of reinfected rabbits there is no preliminary multiplication. Destruction takes place from the beginning, and again as in the other organs, is not complete; a few lingering bacilli persist even 6 weeks after reinfection.

Associated with the immediate destruction in the organs of the bacilli of reinfection there is no evidence of any tuberculous lesions due to the reinfection. This is brought out clearly in the intervals of the fourth and sixth weeks after infection and after reinfection. Although there is a generalized miliary tuberculosis of variable degree

in all the organs of primarily infected rabbits, in the reinfected rabbits no tuberculosis developed in the liver, spleen and bone marrow and the variable lesions in the lung and kidney were undoubtedly residual lesions from the primary infection. Further evidence for the source of the bacilli and the tuberculous lesions in the lung and kidney of reinfected rabbits will be presented in connection with the fate of bovine tubercle bacilli of reinfection.

The Fate of Bovine Tubercle Bacilli of Reinfection in the Organs of Rabbits

In Table II the fate of bovine tubercle bacilli in the organs of infected and of reinfected rabbits as well as the tuberculous changes that they have induced are compared.

In the *lung* of normal rabbits the bovine tubercle bacilli multiply uninterruptedly to the end of the second month, so that by this time the lungs of some rabbits become virtually a pure culture of tubercle bacilli, and as many as 200,000 colonies have been isolated from 10 milligrams of tissue. In the lungs of reinfected rabbits however there is the same lack of a regular tendency to either growth or destruction as was noted above in rabbits reinfected with human tubercle bacilli. Here again extremely variable numbers were isolated. Here, too, the lungs of at least one reinfected rabbit of each interval can be seen to contain such numbers of organisms as can be ascribed to residual bacilli from the primary infection, as in rabbits 43, 45, 46, 48, 51 and 54. Again in the first 2 weeks after reinfection the lesions found in the lungs are unquestionably due to the primary and not to the secondary infection.

More direct evidence was obtained for the origin of these lesions and bacilli, first in the x-ray pictures of the lungs of the rabbits taken just before reinfection, and secondly in the cultural behaviour of these bacilli.

It was found that if a rabbit showed slight or no lesions by x-ray previous to reinfection, slight or no lesions were found in the lungs at autopsy whether the animal was killed 24 hours or 2 months after reinfection. Rabbit 45 showed no lesions detectable by x-ray before reinfection and showed no lesions at autopsy 1 week after reinfection. Rabbit 54 showed no distinct lesions by x-ray previous to the second inoculation and only a few discrete tubercles in the lungs when he was killed 2 months after reinfection.

On the other hand, if the rabbit showed extensive lesions detectable by x-ray before reinfection these were found at autopsy. Rabbit 44 showed extensive pulmonary tuberculosis before reinfection and showed discrete pus-containing cavities when killed 1 week after reinfection. Similarly rabbits 47, 49 and 52, which were killed 2, 4 and 6 weeks after reinfection respectively. Moreover the

TABLE II

Number of Colonies and the Pathological Changes Found in Rabbits Infected, and in Rabbits Reinfected† with 0.01 Mg. B. tuberculosis, Bovine Type

Interval after infection or reinfection	Rabbit No. Inf.	Rabbit No. Reinf.	Lung Inf.	Lung Reinf.	Liver Inf.	Liver Reinf.	Spleen Inf.	Spleen Reinf.	Kidney Inf.	Kidney Reinf.	Bone marrow Inf.	Bone marrow Reinf.	Pathological findings Infected	Pathological findings Reinfected
1 day	29	42	3*	8,300	13	0*	7.3	0	0.3	0	2.3	0.3	Neg.	Tuberculous bronchopneumonia with cavitation; caseous pus in pelvis of one kidney. Few residual pulm. tuberc.; caseous tbc. of medulla of kidneys; tbc. of joints; x-ray free of tbc. before reinfection
	30	43	?	20	3*	11	16*	12	0	405	2.5	11	Neg.	
1 week	31	44	6*	30,000	28	3	69	200	0.2	1,630	18	16	Neg.	Discrete pus containing pulm. cavities. Tbc. of joints; bacteremia? T.B. of human type. Ext. pulm. tbc. before reinfection. 2 or 3 residual tuberc. in both lungs; practically free of tbc. before reinfection by x-ray. Tbc. of one joint
	32	45	6*	70	31	537	8	6	6	0.3	3	0	Neg.	

2 weeks	33	46	1,010	300	150		2,410	1	0.3	0	4	64	0	Minute pulmonary miliary tubercles	Fibrocaseous consolidation of anterior pulm. margins with few discrete pulm. tuberc. over remainder of lung; tbc. of tibia
	34	47	1,200	1,270	380	0.3	1,425	27	0	300	480	0.6		Punctate miliary tubercles of lung, liver, spleen and kidney	Extensive ulcerative pulm. tbc.; miliary tubercles in cortex of kidneys; tbc. of one joint; ext. tbc. before reinfection
4 weeks	35	48	1,080	10*	?	0	466	297	0	10	170	0		Ext. discrete miliary tbc. of lungs; miliary tuberc. in liver, spleen, kidney and bone marrow	Moderate no. of miliary tubercles in lungs; few pinpoint tubercles in kidneys
	36	49	1,000*	6,000	305*	18	1,650	54	12	517	420	3		Numerous pulm. miliary tuberc. with caseation; slight to moderate miliary tbc. of liver, spleen, kidney and bone marrow	Ulcerative caseous pulm. tbc. Cortical and cortico-med. tbc. of kidneys; tbc. of joints; tbc. of both lungs before reinf.; t.b. of human type. Bacteremia?
	50				60*			0.2	0	20		7			Discrete, sparse tubercles in both lungs. With localized lesions in anterior margin and apex of rt. lung. Few cortical tuberc. in kidney; localized lesions in lung before reinfection

† The reinfected rabbits had a primary inoculation of 0.001 mg. B. tuberculosis, human type, about six months before reinfection.

* Adjusted from figures obtained after treatment.

TABLE II—*Concluded*

Interval after infection or reinfection	Rabbit No. Inf.	Rabbit No. Reinf.	Lung Inf.	Lung Reinf.	Liver Inf.	Liver Reinf.	Spleen Inf.	Spleen Reinf.	Kidney Inf.	Kidney Reinf.	Bone marrow Inf.	Bone marrow Reinf.	Pathological findings — Infected	Pathological findings — Reinfected
6 weeks	37	51	12,000	320*	1.3	0	4,000	0	1,530	2	1,575	0	Massive miliary conglom. plum. tbc. with caseation; slight to ext. miliary tbc. in liver, spleen, kidney and bone marrow	Numerous discrete subpleural tuberc. 3–4 mm. in diameter
	38	52	2,100*	2,560	38	0.3	82	0.3	111	130	23	1	Ext. miliary conglom. pulm. tbc. with caseation, with miliary tuberc. in spleen, liver and kidney	Ulcerative pulm. tbc. with discrete tubercles; few cortical tubercles in kidneys. Disseminated pulm. tbc. before reinfection
		53		540*		0		0		3.5		180		Discrete tuberc. uniformly distributed over both lungs; few tuberc. in kidneys and bone marrow; free of tbc. before reinfection
2 mos.	39	54	3,100	260*	130	0	620	0	1,300	0		0	Massive consolidation with conglomerate tuberc. with multiple foci of caseation in both lungs; slight to ext. miliary tbc. of liver, spleen, kidney and bone marrow	Few discrete tubercles in lungs which were practically free of tbc. before reinfection

40	55	6,300*	11,000*	0	0	1,530	1	505	366	690	6*	Massive conglom. miliary tbc. with caseation of both lungs; miliary tbc. of spleen, kidney and bone marrow	Ulcerative pulm. tbc.; tbc. of cortex of left and caseous pus in right kidney
41	56	200,000	1,100	0.7	0.5	2,900	1	1,168	70	3	0.5	Massive caseous pneumonia and conglom. miliary tbc. of both lungs; several tubercles in kidneys and spleen	Numerous discrete subpleural tubercles; few cortical tubercles in kidneys; tbc. of joints

FIGURES I TO III

more extensive the lesions shown by x-ray before reinfection, the more tubercle bacilli were isolated.

Further evidence pointing to the primary infection as the source of the bacilli cultured from the lungs of reinfected rabbits is as follows. It is well known that bovine tubercle bacilli on isolation from the body are dysgonic, that is, that they grow more slowly in the test tubes than human bacilli. Now although both the human and bovine strains of tubercle bacilli used in these experiments grew equally fast when transplanted on glycerol agar in the usual way, the dysgonicity of the bovine strain appears when the bacilli are reisolated from the organs of the rabbit. From the organs of numerous rabbits given a primary inoculation of human bacilli, 86 per cent of the colonies that were finally present after 3 months' incubation were already grown by the fifth week of incubation and all had appeared by the sixth week. On the other hand, from the organs of rabbits given a primary bovine infection, only 25 per cent grew in the first 6 weeks, whereas the great majority appeared between the 8th and 10th week, and a considerable number had not yet appeared before the twelfth week. See figures I and II.

Now from the organs of the rabbits that had been given a primary inoculation of human tubercle bacilli and had been reinfected with bovine tubercle bacilli two distinct groups of organisms were isolated. Of these, 61 per cent appeared within the first 6 weeks after incubation and 39 per cent appeared between the sixth and twelfth week after incubation. Thus according to their rapidity of growth on culture they are seen to be of two types, some behaving as human bacilli, others as bovine bacilli. See figure III.

It appears that the tubercle bacilli isolated from the lungs of all the reinfected rabbits except two, namely rabbits 51 and 53 of the 6-weeks interval, had grown in the first 6 weeks. Thus by far the largest number of tubercle bacilli isolated from the lungs of the rabbits reinfected with bovine bacilli behaved not like the bovine organism but like the human tubercle bacillus and therefore probably have been derived from the primary infection.

Thus the x-ray evidence before reinfection and the cultural behavior of the bacilli recovered, as well as the very low numbers of the organisms found in at least one rabbit at each interval after reinfection, as contrasted with their continuously increasing and finally tremendous numbers after primary infection, all argue strongly for the conclusion that the bovine bacillus does not grow as a rule in the lungs of reinfected rabbits.

That some growth of bovine bacilli of reinfection can take place in the lungs, though unquestionably a restricted one, is probably shown in rabbits 51 and 53. Rabbit 51 had about 320 colonies in 10 mg. of lung, which appeared in culture on the 12th week of incubation. Rabbit 53 showed no detectable lesions in the lung by x-ray before reinfection, yet when this rabbit was killed 6 weeks after reinfection there were discrete uniformly distributed tubercles in both lungs and the organism cultured from this rabbit appeared on the 8th week of incubation.

That the growth of bovine bacilli of reinfection in the lungs of even these rabbits

is greatly suppressed as compared with bacilli from a primary infection is seen from the fact that the average number of colonies recovered from 2 normal rabbits killed 6 weeks after infection was 7050, in contrast to the average of only 430 colonies derived from these two rabbits.

It is noteworthy that this, the only evidence obtained of multiplication of tubercle bacilli in the lungs of reinfected rabbits, was found in rabbits having practically no residual lesions from the primary infection.

In the *liver* of normal rabbits multiplication of the bovine bacilli is slow at first, reaches its height in the fourth week and thereafter declines rapidly. In the liver of reinfected rabbits fewer bacilli are isolated even 24 hours after the second infection than are found in primarily infected rabbits, showing a tendency to immediate destruction. Thereafter, as a rule, only a very few organisms are found, showing again as after reinfection with human bacilli, that destruction is never quite complete.

The presence of from 18 to 31 colonies in the liver in 3 rabbits of this group requires some explanation. Of these, rabbit 44 of the first-week interval, and rabbit 49 of the fourth-week interval, had extensive pulmonary tuberculosis before reinfection. At autopsy both showed an extensive ulcerative tuberculosis of both lungs with very large numbers of tubercle bacilli. Both were apparently suffering from a bacteremia derived from the extensive pulmonary foci, as evidenced by the tuberculosis of the joints, the distribution of bacilli in the liver and spleen, which was like the distribution following immediately upon a primary inoculation, and the cultural characteristics of these organisms, which were of the human type; unfortunately no blood cultures were made. These colonies therefore are due not to a growth of the bovine tubercle bacilli of reinfection but to a flooding of the circulation with human tubercle bacilli from the old foci in the lungs and their filtration in the various organs. In rabbit 45, however, the 31 colonies isolated are possibly due to an actual multiplication of the bovine bacilli of reinfection, for they were culturally of the bovine type. It is noteworthy that this rabbit was free of tuberculosis before reinfection and showed only 2 small residual tubercles in the lung when it was killed one week after reinfection.

In the *spleen* of normal rabbits the bovine bacilli multiply at once, and continue to multiply to the sixth week; they are then slowly reduced in number, although many are still present at two months. In the spleen of the reinfected rabbits the tubercle bacilli are destroyed from the very first, so that even 24 hours after reinfection they are already less in number than after a primary infection with bovine bacilli. Thereafter only an occasional organism is isolated, except in rabbits 44 and 49, where there are some tubercle bacilli due to the bacteremia from the lungs in this organ as in the liver.

In the *kidneys* of normal rabbits the bovine bacillus accumulates very slowly at first and continues persistently with scarcely any abatement to the end of the experiment. In the kidneys of reinfected rabbits on the other hand there is the same irregularity as was noted in the lungs. Here again there is at least one

rabbit at each interval with only few residual tubercle bacilli and with no evidence of any multiplication even 2 months after reinfection. There is however another group of rabbits in which variable and at times considerable numbers of tubercle bacilli have been isolated from the kidneys. These all behaved in culture like the human type, and like those in the lungs of the same rabbits are most probably due to bacilli from the primary infection. It is noteworthy that the kidneys of rabbit 44, from which the largest number, 1630 colonies, of tubercle bacilli were isolated, showed no tuberculosis at all. Are these bacilli explained by bacteremia in this rabbit and are they being excreted by the kidney?

In the *bone marrow* of normal rabbits the bovine tubercle bacillus multiplies slowly at first. This continues to the sixth week when their numbers slowly decline. At two months considerable numbers of tubercle bacilli are still present. In the bone marrow of reinfected rabbits only occasional tubercle bacilli can be isolated; they are destroyed without any preliminary growth.

There was only one exception to this rule, rabbit 53, in which unquestioned though restricted growth took place, for 180 colonies were isolated from the bone marrow of this rabbit 6 weeks after reinfection. These were of the bovine type and could scarcely be residual bacilli from the primary infection. It is noteworthy that this rabbit also showed restricted multiplication of the bacilli of reinfection in the lungs, and, as was stated above, was free of tuberculosis before reinfection.

Associated with the immediate destruction of the bovine tubercle bacilli of reinfection in the organs of most of the rabbits, there was no evidence of any lesions due to the bacilli of reinfection; whereas the normal animals similarly infected showed a massive pulmonary miliary tuberculosis with extensive to moderate tuberculosis in the liver, spleen, kidney and bone marrow. In no reinfected rabbit was there any tuberculosis of the liver and spleen and in only one were there a few tubercles in the bone marrow. In the lungs of reinfected rabbits the lesions were different in character from those found in infected rabbits. The latter showed consolidation of the entire lung by a uniformly distributed massive miliary tuberculosis; in the former, the pulmonary lesions were often very unevenly distributed, irregular in size, extremely few in number or they consisted of more or less extensive pus cavities, such as are found in rabbits that are permitted to live a long time after a single injection of human tubercle bacilli. These lesions were present in the rabbits before reinfection, as the x-ray photographs showed. There were only 2 reinfected rabbits in which some pulmonary tuberculosis developed later, which however was very slight in comparison with that in the infected rabbits.

SUMMARY AND DISCUSSION

In agreement with previous work (6) it was found that in normal rabbits after a primary inoculation of 0.01 mg. of human tubercle bacilli the organism shows a preliminary growth, which commences at once in all the organs, and reaches its height in the second week in the liver, spleen, and bone marrow; thereafter destruction begins, and is practically complete by the sixth week in the liver and bone marrow and less complete in the spleen. In the lung and kidney the largest number are isolated in the fourth week and thereafter there is a tendency to destruction, but to destruction far less complete than in the other organs, for even two months after infection large numbers of tubercle bacilli persist. Associated with this multiplication of the human tubercle bacilli were found extensive or moderate tuberculous lesions in the lung and kidney with moderate or slight changes in the liver, spleen and bone marrow.

In contrast to these observations, it was found that in rabbits reinfected with the same quantity of human tubercle bacilli, the organism was destroyed immediately without any preliminary multiplication in the liver, spleen and bone marrow, though a few bacilli persisted in these organs even two months after reinfection. Neither could definite evidence of multiplication of the tubercle bacilli of reinfection in the lung and kidney of these rabbits be obtained; although variable and at times very large numbers of tubercle bacilli persisted in these organs, probably due to the primary infection. Nor were tuberculous changes found in any of the organs due to reinfection.

Again in rabbits that received a primary inoculation of 0.01 mg. of bovine tubercle bacilli the organism multiplied in all the organs. This multiplication reached its height in the liver, spleen and bone marrow between the fourth and sixth week, instead of in the second week as with human bacilli. Thereafter destruction was practically complete in the liver and much less complete in the spleen and bone marrow; in the lung and kidney multiplication continued unabated to the second month. Associated with these bacteriological data, there developed a massive pulmonary tuberculosis with extensive or moderate disease in the kidney, spleen, bone marrow and liver.

In rabbits reinfected with the same quantity of bovine tubercle bacilli after a primary infection with human tubercle bacilli, the

organism, in all but a few instances, was destroyed immediately in the liver, spleen and bone marrow. Here again, with the bovine type as with the human type, after reinfection as after primary infection, the destruction was not quite complete and a few isolated bacilli persisted even two months after reinfection. As to the lung and kidney, evidence was obtained that the variable and at times very large numbers of tubercle bacilli that were found in these organs were of human type in their cultural characteristics, and that in these organs also, the bovine tubercle bacilli of reinfection failed to grow. There was also as a rule complete absence of any tuberculous lesions in the liver, spleen and bone marrow. Nor were there any tuberculous changes found in the lung and kidney due to the reinfection, but those variable residual lesions that were found were present before reinfection, as shown in the lung by x-ray photographs. There were a few instances in which restricted multiplication of the virus took place and slight tuberculous lesions developed after reinfection in rabbits in which the primary lesions had all but disappeared.

Thus in rabbits having considerable residual lesions from a primary human infection the tubercle bacilli of reinfection, whether human or bovine, are destroyed immediately though incompletely without any preliminary multiplication. Yet these rabbits, which so efficiently destroy the more virulent bovine bacilli of reinfection introduced from without, at times harbor tremendous numbers of human tubercle bacilli in the old lesions of the lung and kidney.

Parallel with the bacteriological and pathological studies of these rabbits, the serum of some of them was studied for the presence of circulating antibodies by McCutcheon, Strumia, Mudd (S), Mudd (E. B. H.) and Lucké (7). These investigators found that on primary infection there was only a slight and slowly developing production of agglutinating and phagocytosis-promoting antibodies whereas, in the reinfected rabbits, which showed an immediate destruction of the bacilli, these antibodies rose promptly and in relatively large amount. They suggest that the prompt rise of antibodies in reinfected animals may play a rôle in the immunity to tuberculosis.

In the previous study, which was preformed in 1927, emphasis was placed upon the comparatively slower original rate of growth of the bovine bacillus in the rabbit as compared with that of the human type,

and in this delay was seen a partial explanation of the greater virulence of the bovine organism for this species. The more rapid the original growth the more rapid was the following destruction. In this series of experiments, which were performed early in 1929, although the delayed destruction of the bovine type in the rabbit was amply confirmed, the original rate of growth is not any slower than the human type. Coincident with this increasingly more rapid rate of growth of the bovine bacillus in the body is a gradual decrease in the virulence of the organism. In 1925 and 1926 0.01 mg. of this bovine strain regularly killed rabbits in about 30 days after intravenous inoculation (8), but in this experiment none of the 3 rabbits died within 65 days after a similar infection and 2 rabbits were still living 110 days after injection.

CONCLUSIONS

1. In the presence of a certain amount of residual primary lesions, human or bovine tubercle bacilli of reinfection are destroyed in all the organs of rabbits without any preliminary multiplication. This destruction is not quite complete; a few organisms persist even two months after reinfection.

2. No macroscopic tuberculous lesions due to the reinfection develop in these rabbits.

3. With the practical disappearance of the primary lesions and their enclosed organisms, restricted multiplication of the bovine bacillus of reinfection takes place but not the extensive growth of the virus observed in rabbits after a primary infection.

4. In these rabbits slight tuberculous lesions develop as compared with the massive tuberculosis of primarily infected rabbits.

5. The human tubercle bacillus of reinfection does not multiply at all, nor does it cause any lesions, even when the lesions of the primary infection have almost disappeared.

6. Although the rabbit destroys efficiently considerable quantities of bovine tubercle bacilli of reinfection introduced from without it may harbor innumerable human bacilli in the residual primary lesions of the lung and kidney.

I take this opportunity to express my indebtedness to Dr. Jules Freund for many valuable suggestions during the execution of this work.

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STUDIES ON THE DISSOCIATION OF THE HOG CHOLERA BACILLUS

III. Active Immunization with R Forms

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In previous papers (1), (2) studies on the dissociation of the so-called hog cholera bacillus were presented. It was found that at least 4 distinct forms of the bacillus existed —the "normal" or MS strain and its 3 variants, MR, NS and NR. From immunological tests it was apparent that the symbols MS, MR, NS and NR corresponded to the symbols OH, ØH, O and Ø, respectively, used by previous investigators. The virulence of the S forms, as in other bacterial species, was greater than that of the R forms. Both MS and NS forms were virulent for hamsters and rabbits. The NR form, however, usually gave rise to fever or abscess formation and occasionally caused the death of these animals. In attempts to induce reversion from one form to another, it was found that the MR form, which, as such, consistently lacked virulence, was under certain conditions the most unstable of the forms. It reverted either to its prototype, the virulent MS form, or dissociated further into the NR· form (2). This observation was of particular interest and will be referred to presently.

It is of importance to determine for practical purposes whether the killed, or especially the living avirulent R forms, are of value as prophylactic vaccines. It is also desirable to determine which of the antigenic components, the H, O or the Ø, separately or in combination, are responsible for the stimulation of immunity. In this paper studies are reported which were made to determine the immunizing power of the avirulent forms of the hog cholera bacillus isolated in this laboratory.

Many studies have already been made in regard to the virulence or avirulence of bacteria used as antigens. Rowland (3), working with plague bacilli, Weber (4), with typhoid bacilli, and Griffith (5), with pneumococci, all agreed that avirulent forms of the respective bacteria were unsuitable for the production of efficient vaccines or antisera. The Army Council (6), however, reported that virulence is not an essential characteristic of organisms used for the production of vaccine. Tillett (7) also found that some degree of immunity against S strains could be established in rabbits with killed R Pneumococci, although antiserum produced by the injection of R forms into rabbits was valueless in passive protection of mice.

Other investigations have shown that animals inoculated with the living avirulent form were resistant to the virulent form of the same organism. Manninger (8) found this to be true with B. avisepticus in mice, De Kruif (9) with the B. lepisepticum, and Cowan (10) with the streptococcus in mice. With considerable difficulty, White (11) succeeded in immunizing rabbits against the hog cholera bacillus by vaccinating them with killed S and R organisms as well as with living R forms alone.

Much attention has also been devoted to the comparative antigenic power of the various antigenic components present in bacterial vaccines. Felix and Olitzki (12) and Arkwright (13) believed that the "O" antigen was chiefly responsible for the stimulation of immunity, and according to the latter author the presence of the H or $\emptyset$ (rough O) element was unimportant. Springut (14) working with B. typhi murium, however, concluded that animals immunized with both H and O components have a higher degree of protection than those immunized with O antigen alone. Similar observations were made by Ibraham and Schütze (15) who showed that vaccines containing both H and O elements were most effective. "O" vaccines stimulated slight protection and vaccines containing H or $\emptyset$ alone or together were without value.

Method

The hamsters (Cricetulus griseus) and rabbits used in these experiments were all derived from the same source and were kept under similar conditions for at least a month before use. In all experiments animals of similar weight were used. Before use the blood of each rabbit was tested and found free from agglutinins for hog cholera bacilli and for rabbit typhoid bacilli.

Vaccine.—24 hour old agar slant cultures of the 4 forms MS, MR, NS and NR of the hog cholera bacillus were suspended in 0.1 per cent sodium chloride solution and standardized by counting and diluting. The organisms were killed by heating to 56°C. for 30 minutes. Living vaccines were prepared by suspending the growth from a 24 hour old broth culture in 0.1 per cent saline solution.

Immunization of Animals.—Hamsters were immunized by injecting vaccine subcutaneously on one side of the dorsal surface once or several times at 7 day intervals. When the animals were tested for immunity, the test organisms were

inoculated subcutaneously on the opposite side of back 7 to 24 days after the last injection. Rabbits were immunized in the same way except that the ventral surface was used. For immunization and for immunity tests the desired amount of vaccine or test organisms were suspended in 1 cc. of 0.1 per cent saline solution before injection.

Cultures on China blue rosalic acid agar plates were made from the spleens of all animals dying during immunization or after the immunity tests. The organisms recovered were identified in the usual way.

After the test inoculation all animals were observed for 30 days. The temperature of the rabbits was taken daily. At the end of 30 days all of the survivors were killed and in most cases cultures of the spleen were made.

EXPERIMENTAL

Vaccination with Killed Organisms.—Preliminary experiments indicated that the injection of killed organisms did not confer immunity against virulent hog cholera bacilli in rabbits. Further efforts were then made to immunize hamsters by the same method.

40 hamsters were divided into 4 groups of 10 each. Each group was immunized with one of the 4 forms MS, MR, NS and NR by injecting 4 increasing doses (40, 100, 500 and 1000 million) of heat killed bacilli at 7 day intervals. 10 days after the last injection, all the hamsters, together with 10 unvaccinated control animals, were inoculated subcutaneously with .0001 cc. of a culture of virulent MS bacilli.

Results.—Vaccines consisting of heat killed MS, MR and NR forms were without effect. 8 of the hamsters vaccinated with the MS form, 9 with the MR form and 10 with the NR form died within 2 weeks. 9 of the controls died. The heat killed NS vaccine, however, appeared to confer some degree of immunity since only 5 of the 10 hamsters vaccinated with this form succumbed.

Vaccination with Living MR and NR Forms.—

Two groups of 15 hamsters each were given single subcutaneous injections of .01 cc. of living broth culture of the MR and the NR forms respectively. One hamster from each group died. No growth was obtained from the spleen of the one vaccinated with the MR form, but the NR form was recovered from the animal which was inoculated with that form. 11 of the 14 animals which survived injection of the NR form developed an abscess at the site of inoculation.

Two weeks after vaccination all of the hamsters together with 10 unvaccinated ones were given .0001 cc. of the living MS form subcutaneously. The results are shown in Table I.

Results.—More than 50 per cent of the vaccinated animals survived while all of the control animals died within 10 days. It appears therefore that the living organisms of both MR and NR forms confer active immunity.

The experiment was repeated by immunizing 20 hamsters with a smaller dose (.005 cc.) of living NR bacilli and repeating the injection 20 days later. 2 animals died after the second injection and NR bacilli were recovered from the spleen. The remaining 18 animals and 10 controls were inoculated with .001 cc. and .0001 cc. respectively of a broth culture of the MR form 24 days after the second vaccination.

TABLE I

Immunization of Hamsters with 1 Dose of .01 Cc. Broth Culture of Living MR and NR Hog Cholera Bacilli

The animals were inoculated subcutaneously with .0001 cc. of 24 hour broth culture of MS form of hog cholera bacillus 2 weeks later.

Immunized with	No. used for test	No. died in days after test-dose				Percentage of survivors after 30 days
		1–5	6–10	11–15	16–20	
MR	14	1	2	1	2	57
NR	14	0	1	4		64
Unvaccinated (control)	10	3	7			0

Results.—9 of the 10 controls died while 14 out of 18 or 77 per cent of the vaccinated animals survived. All of the animals which recovered were killed after 30 days and spleen culture were made. The MS form was recovered from the 1 control animal and from 8 of the 14 vaccinated hamsters. No growth was obtained from the remaining 6.

Cross Immunity with B. paratyphosus "C."—It has already been shown by TenBroeck (16) and confirmed by Schütze (17) that a cross immunity exists between the hog cholera bacillus and B. paratyphosus "C" (Hirschfeld). Experiments were then made to determine the immunity of hamsters to B. paratyphosus "C" after vaccination with the living R forms of hog cholera bacilli.

20 hamsters were inoculated subcutaneously with .01 cc. of a living broth culture of the MR form. Later, 2 doses, .005 cc. and .01 cc. of a living broth culture of

NR bacilli were given subcutaneously, 7 days intervening between each injection. None of the animals died during the immunization period. The immunity was tested 14 days later by injecting half of the hamsters with virulent hog cholera bacilli and the other half with B. paratyphosus "C." The results are shown in Table II.

Results. —It is evident that hamsters vaccinated with R forms of hog cholera bacilli are immune to B. paratyphosus "C" as well as to the MS form of the homologous race.

Vaccination with R. Paratyphoid Bacilli.—An experiment the reverse of the preceding was next carried out.

TABLE II

Hamsters Immunized Subcutaneously with 3 Doses of Living MR and NR Forms of Hog Cholera Bacilli: MR .01 Cc., NR .005 Cc. and .01 Cc. at 7 Day Intervals

The animals were inoculated subcutaneously with MS form of B. paratyphosus "C" or MS hog cholera bacillus 14 days after the last immunizing dose.

Immunized with	No. used	Test-dose	No. died	Percentage of survivors after 30 days
Hog cholera bacillus MR and NR	10	B. para. "C", MS, .001 cc.	0	100
Unvaccinated (control)	10	B. para. "C", MS, .001 cc.	8	20
Hog cholera bacillus MR and NR	10	Hog cholera bacillus MS, .0001 cc.	2	80
Unvaccinated (control)	8	Hog cholera bacillus MS, .0001 cc.	8	0

A motile rough (MR) variant was derived from the "normal" MS paratyphoid "C" bacillus. The MR form was found to be avirulent for hamsters. 20 hamsters were then immunized with this form by injecting subcutaneously at weekly intervals one dose of 400 million killed bacilli followed by .0001 cc. and .002 cc. of the living broth culture. None of the animals died during immunization. 10 days after the last dose, 10 of the hamsters were inoculated with MS hog cholera bacilli and 10 with MS paratyphoid "C" bacilli. 20 unvaccinated control hamsters divided into 2 groups of 10 each were also inoculated with MS hog cholera and paratyphoid "C" bacilli respectively.

Results.—The results after 30 days were practically the same as those of the preceding experiment. Vaccination with MR paraty-

phoid "C" bacilli conferred immunity against both virulent hog cholera bacilli and the MS form of the homologous race.   80 per cent of the vaccinated animals survived the injection of hog cholera bacilli while only 10 per cent of the controls did.   All of the vaccinated animals survived inoculation with MS paratyphoid "C" bacilli and all of the controls died.

The foregoing experiments are summarized in Table III.   The results of the vaccination of hamsters with the living R form of both

TABLE III

Summary of Immunization Experiments in Hamsters with Living R Culture against S Hog Cholera and Paratyphoid "C" Bacilli

Immunized with		Tested with	No. used	No. died	Percentage of survivors after 30 days
Hog cholera bacillus, MR		Hog cholera bacillus, MS	14	6	57
"	NR	"	14	5	64
"	NR	"	18	4	77
"	MR & NR	"	10	2	80
"	MR & NR	B. para. "C" MS	10	0	100
B. para. "C" MR		"	10	0	100
"		Hog cholera bacillus, MS	10	2	80
Total....................		Hog cholera bacillus, MS or B. para. "C," MS	86	19	78
Unvaccinated (control)		Hog cholera bacillus, MS or B. para. "C," MS	68	63	7

hog cholera and paratyphoid "C" bacilli are striking.   Of the 86 animals vaccinated with living R forms, 67 survived the subsequent inoculation of virulent S forms of bacilli, either of the homologous or the heterologous race.   Only a small number (7 per cent of 68 animals) of the unvaccinated control hamsters survived although in many instances they received much smaller doses of virulent bacilli than the vaccinated animals.

Experiments with Rabbits.—As mentioned previously, vaccination of rabbits with killed hog cholera bacilli was unsuccessful.

TABLE IV

Immunization of Rabbits with Killed S or with Living R Hog Cholera Bacilli against the Virulent MS Form

S = survival, D = death, F = fever, 40–41°C.

Rabbit No.	Immunization			Test				Result
	Variant	Treatment	Dose	Route	Days after last injection		Dose	
1	NS	Heated 100°C., 60'	7 doses, 50-500 million at 7 day interval	I. V.	7		.0001	D. in 11 days
2	NS	Heated 56°C., 30'	"	"	"		"	D. in 10 days
3	NR	"	"	"	"		"	D. in 8 days
4	MS	"	"	"	"		"	D. in 8 days
5	MR	Living	1 dose, 0.1 cc.	Subc.	14		.1	D. in 5 days
6	NR	"	"	"	"		"	S., F. 19 days
7	MR	"	"	"	20		.001	S., F. 9 days
8	NR	"	"	"	"		"	S., F. 9 days
9	MR & NR	"	3 doses, MR .05 cc., NR .025 cc. and 1 cc. at 7 day intervals	"	14		"	S., F. 12 days
10	"	"	"	"	"		"	S.
11	"	"	"	"	"		"	S., F. 15 days
12		Unvaccinated (control)					.1	D. in 7 days
13		"					.001	D. in 4 days
14		"					.0001	D. in 6 days
15		"					.000001	D. in 7 days

4 animals were given 7 intravenous injections at 7 day intervals of large doses (50 million to 500 million) of the heat killed bacilli. All succumbed within 8 to 11 days after the inoculation of .0001 cc. of the MS form.

7 rabbits were then inoculated subcutaneously with 1 or more doses of living R cultures as indicated in Table IV. Some of the animals developed fever of 40°C. lasting from 1 to 5 days after the first dose and lost weight. No abscess formation was observed. 2 or 3 weeks after the last injection, they were inoculated subcutaneously with the MS form.

As shown in Table IV only 1 of the animals died after receiving a large dose, .1 cc., of a broth culture. All of the controls, including the one which received only .000001 cc. of the culture, died within 7 days. Most of the survivors, however, developed fever 2 to 4 days after the inoculation which lasted from 9 to 19 days, and lost weight. With the disappearance of fever they regained weight and recovered.

DISCUSSION

It has been demonstrated that the vaccination of hamsters and rabbits with living R forms of hog cholera bacilli and paratyphoid "C" bacilli elicits a definite immunity against the virulent S forms of both kinds of bacilli. Further work is, of course, necessary to determine the practical value of using living R forms of various bacteria as prophylactic vaccines. It should be borne in mind that in the case of the hog cholera bacillus, the NR cultures are not absolutely innocuous. NR bacilli occasionally cause death and frequently give rise to abscess formation in hamsters. Both NR and MR bacilli cause illness and fever in rabbits. Furthermore, as alluded to before, it was shown in a previous paper (2) that MR bacilli may revert to the virulent MS form *in vitro* and *in vivo*. The reversion to the MS form *in vivo* occurred after large doses had been injected intraperitoneally. In the present investigation the MR forms were injected subcutaneously and no reversion was observed.

In regard to the comparative immunizing power of the various antigenic components in the vaccine, the results of these experiments are at variance with those of certain other investigations. It was found that immunity was stimulated by both ØH (MR) and Ø (NR) forms of the living bacilli.

SUMMARY

1. Heat killed organisms of the MS (OH), MR (ØH), NS (O) or NR (Ø) forms of hog cholera bacilli failed to confer immunity, at

least to any considerable degree, in hamsters or rabbits against virulent hog cholera bacilli.

2. Hamsters and rabbits were successfully immunized against virulent MS bacilli by vaccination with living MR (ØH) and NR (Ø) forms.

3. Hamsters were immunized against MS hog cholera bacilli with living R paratyphoid "C" bacilli and conversely against MS paratyphoid "C" bacilli with living R hog cholera bacilli.

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ANAPHYLACTIC SHOCK WITH THE PARTIAL ANTIGEN OF THE TUBERCLE BACILLUS

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The relationship of the so-called specific soluble substance to acute anaphylactic shock has been only recently determined. This appears somewhat remarkable in view of the well recognized precipitogenic action of the partial antigen *in vitro* together with the long-observed correlation of high precipitin titre with a serum's effectiveness as a sensitizing agent—facts which a priori almost force the assignment to the specific polysaccharide of an essential function in the anaphylactic mechanism. Tomcsik (1) in 1927, working with the carbohydrate of *B. lactis aerogenes,* showed that guinea pigs passively sensitized with the homologous antiserum died acutely when injected with the specific soluble substance. In addition, he found that the isolated uterus of a sensitized guinea pig reacted to exceedingly small quantities of the material. These observations were extended by Tomcsik and Kurotchkin (2) to include the carbohydrates obtained from the pneumobacillus and from yeast. In 1929 Avery and Tillett (3) confirmed the work of these authors by producing with the specific carbohydrate of the pneumococcus anaphylactic shock in guinea pigs treated with antipneumococcus rabbit sera corresponding to Types I, II and III. Moreover, they showed that the type specificity of the carbohydrate held rigidly in its anaphylactogenic rôle. Noted by them also was the interesting and as yet unexplained fact that antipneumococcus horse serum was incapable of sensitizing.

On the basis of these facts it appears probable that in every case in which a specific carbohydrate can be obtained from a complete antigen complex capable of producing antibodies *in vivo* anaphylactic

shock can be elicited under suitable conditions by means of the partial antigen.

The application, then, of what appears to be a general principle to the problem of hypersensitiveness in tuberculosis and its relation to true anaphylaxis seemed to offer a new instrument in the study of the mechanism involved. The experiments reported in this communication were designed mainly to confirm in respect to the carbohydrate of the tubercle bacillus the work of the previous authors. Only in a preliminary fashion do they touch upon the problem of allergy in tuberculosis.

The specific carbohydrate used in this work was derived according to the process devised by Mueller (4) from O. T. produced by a human strain (H 37— Saranac) grown on a synthetic medium. The substance contains 0.3 per cent nitrogen and a very slight trace of phosphorus. 3 per cent ash containing a trace of copper is present. It does not reduce Benedict's solution, but gives a very strong·alpha-naphthol test for carbohydrate. It gives no precipitate with picric, tannic or phosphotungstic acid, not with lead acetate, neutral or basic copper sulfate, uranium nitrate, silver nitrate, mercuric nitrate, nor with safranine. Optical rotation is determined on a 3 per cent aqueous solution in a 1 cm. tube: $[\alpha]_D = +17.3°$. It does not give a skin test in tuberculous animals or animals rendered hypersensitive to tuberculin by injection of killed tubercle bacilli (2). It gives a ring test with the homologous antiserum in a dilution of 1:2,000,000.

Since the results of all who have worked with various bacterial carbohydrates show that such substances are not antigenic when introduced into the living organism, it was not considered essential again to demonstrate a lack of antigenicity with this material.

Passive Sensitization

To obtain an antiserum with which passive sensitization could be carried out, a rabbit was given four injections of a saline suspension of dead tubercle bacilli (H 37 — 26.5 mg. dry weight at each injection) grown for 8 weeks on 5 per cent glycerine bouillon and killed by heating at 80°C. for an hour. The injections were done on every fourth day. With varying dilutions of a commercial O. T. (Mulford), the pure serum taken 4 days after the last injection showed a distinct ring in 1:10,000. Unfortunately, owing to an accident, only sufficient serum was obtained to sensitize 3 guinea pigs. Each pig was given 2 cc. of the rabbit serum intraperitoneally. The results obtained by intravenous (ear vein) administration of the specific carbohydrate are shown in Table I.

TABLE I

Guinea pig No.	Sensitizing dose anti-Tb. rabbit serum	Shocking dose of SSS (Tb)	Hours elapsed between sensitizing and shocking dose	Results
	cc.	mg.		
1	2	2	24	Acute symptoms of shock-death in 3 min. Autopsy showed typical pulmonary emphysema—heart still beating
2	2	2	98	Symptoms within 1 min. Dyspnoea, violent scratching, urination, defecation. Extreme prostration. Recovery after 15 minutes
3	2	2	98	Typical and severe symptoms within 1 min. Extreme dyspnoea with convulsive movements, marked prostration. Slow recovery

Normal animals treated with the same amount of carbohydrate showed no symptoms. Because of the small number of animals represented, a second anti-Tb. rabbit serum was prepared and the experiment repeated.

TABLE II

Guinea pig No.	Sensitizing dose anti-Tb. rabbit serum	Shocking dose of SSS (Tb)	Hours elapsed between sensitizing and shocking dose	Results
	cc.	mg.		
4	2	2	16	Death in 4 min. Autopsy typical
5	2	2	16	Symptoms of severe shock. Slow recovery
6	2	0.5	16	Death in 3 min. Autopsy typical
7	3	1	24	Death in 4 min. Autopsy typical

The rabbit serum used in the experiment shown in Table II gave a precipitin titre against O. T. of 1:10,000. It also gave a definite precipitin titre against the specific soluble substance with a dilution of 1:128 of the serum.

Active Sensitization

The previous workers in this field have dealt only with animals passively sensitized with immune rabbit serum. To study effectively the relation between anaphylaxis and tuberculin allergy it seemed

desirable to produce acute shock in guinea pigs actively sensitized with the tubercle bacillus. If an animal could be obtained which was simultaneously sensitive to intracutaneous inoculation of tuberculin

TABLE III

Guinea pig No.	Sensitizing dose dead H 37, heat killed	Shocking dose of SSS (Tb)	Interval between sensitizing and shocking dose	Results
			days	
8	2.1 mg. every third day— three doses	2 mg. i.v. (ear vein)	38	Symptoms of shock within 2 min. Scratching, dyspnoea, followed by slow recovery
9	Same dose	2 mg. i.v. (ear vein)	38	Symptoms of shock within 2 min. Violent scratching, dyspnoea, urination followed by marked prostration and recovery
10	Same dose	2 mg. i.v. (jugular vein)	42	Slight symptoms—inconclusive
11	Same dose	2 mg. i.v. (jugular vein)	42	Slight symptoms—inconclusive
12	Same dose	2 mg. i.v. (ear vein)	42	Practically no symptoms
13	Same dose	2 mg. i.v. (ear vein)	44	Immediate symptoms. Scratching dyspnoea, followed by a period of prostration. Recovery
14	Same dose	2 mg. i.v. (ear vein)	45	Immediate symptoms of slight shock. Scratching, dyspnoea, etc. Recovery
15	Same dose	2 mg. i.v. (ear vein)	45	Immediate symptoms of shock. Restlessness, scratching, dyspnoea, prostration. Recovery
16	Same dose	2 mg. i.v. (ear vein)	45	Slight symptoms. Inconclusive
17	Same dose	2 mg. i.v. (ear vein)	45	No symptoms

In this series only 50 per cent of the animals showed definite symptoms of shock with the amount of carbohydrate administered. The poor degree of sensitivity observed can probably be attributed to the rather long interval between sensitization and testing.

and to the specific carbohydrate as indicated by acute shock, the effect of anaphylactic desensitization on the intensity of the skin reaction might be accurately observed.

Having on hand a series of guinea pigs weighing from 350 to 400 grams which had received intraperitoneal injections of dead tubercle bacilli, an experiment was undertaken to determine the anaphylactic sensitivity of these animals to the specific carbohydrate. The results are shown in Table III.

TABLE IV

Guinea pig No.	Sensitizing dose heat killed strain H 37 (Tb)	Shocking dose of SSS (Tb)	Interval between last sensitizing and shocking dose	Results
			days	
18	2 6 mg. every 3rd day— three doses	2 mg. SSS (ear vein)	24	Symptoms of acute shock in 1 minute. Marked dyspnoea, scratching, convulsive movements, followed by death in 9 minutes
19	Same dose	2 mg. SSS (ear vein)	24	Immediate symptoms of acute shock. Marked dyspnoea, urination, convulsive movments. Profound prostration and slow recovery
20	Same dose	2 mg. SSS (ear vein)	24	Immediate symptoms of acute shock. Marked dyspnoea, scratching, urination, convulsive movements. Prostration followed by slow recovery
21	Same dose	3 mg. SSS (ear vein)	24	Immediate symptoms of acute shock (within 1 min.). Dyspnoea, scratching, urination, convulsive movements. Prostration followed by death in 8 minutes
22	Same dose	3 mg. SSS (ear vein)	24	Symptoms of shock within 1 minute. Dyspnoea, scratching, convulsions, followed by death in 5 minutes

Another lot of guinea pigs were given intraperitoneal injections of dead tubercle bacilli. These animals were older and heavier than those used in the previous experiment (500–600 grams). When tested with the carbohydrate, the results shown in Table IV were obtained.

It is clear from the foregoing experiments that acute lethal shock

g. Pig # 21-b
Left Horn
8/6/29

5 mg SSS(TB)
in 100 cc bath.
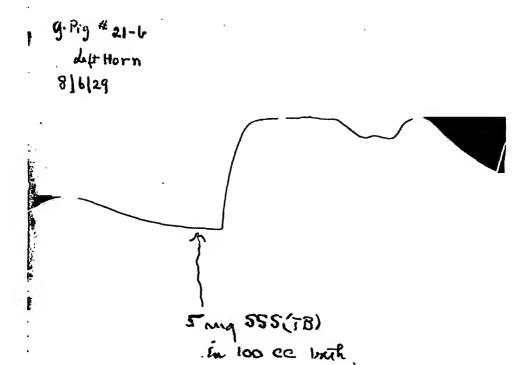
FIG. 1. On August 5, 1929, Guinea Pig 21-b received 2 cc. of anti-Tb. rabbit serum intraperitoneally. 24 hours later the isolated uterus of this pig gave the reaction recorded above when 5 mg. of the specific soluble substance was added to the bath.

g. Pig # 22-b
Left Horn
8/7/29

2 mg SSS (TB)
in 2500 bath
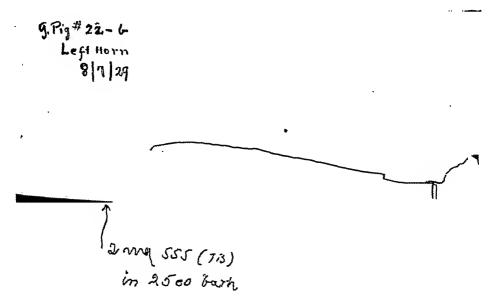
FIG. 2. On July 5, 9, and 12, 1929, respectively, Guinea Pig 22-b received 3 mg. of dried tubercle bacilli intraperitoneally. On August 7 the isolated uterus gave the reaction recorded above when 2 mg. of the specific soluble substance was

can be produced by the specific carbohydrate of the tubercle bacillus in guinea pigs either actively sensitized with the bacteria themselves or with an anti-Tb. rabbit serum of high precipitin titre.

TABLE V

Guinea Pigs Actively Sensitized with Dead Tubercle Bacilli

Guinea pig No.	Dose O. T. intracutaneous	Interval between tuberculin test and shock	Reading of tuberculin reaction No. 1 24 hours after injection	Interval between tuberculin test No. 2 and shock	Reading of tuberculin reaction No. 2 24 hours after injection with O. T.
23	0.1 cc. 1:10 dilution (Mulf)	11 days	+++ Marked necrosis	1 hour	Reading at 48 hours —Redness, edema, central blanching, slight necrosis
24	Same dose	4 days	++ Induration with necrotic centre	18 hours	Reading at 48 hours —Redness, edema, central blanching, slight necrosis
25	Same dose	16 days	+ Marked redness and edema, slight blanching	Not done	Not done
26	Same dose	12 days	++ Blanching	Not done	Not done
27	Same dose	12 days	± Redness and slight edema	1 hour	Reading at 48 hours —Redness,—a weak reaction
28	Same dose	8 days	+ No necrosis. Redness and edema	24 hours	+ Redness and swelling. Slight blanching
29	Same dose	24 days	± Weak reaction		++ Central blanching
30	Same dose	11 days	+ Slight blanching		++ Redness, swelling and blanching
31	Same dose	24 days	+ Blanching	1 hour	+ Redness, swelling and slight blanching
32	Same dose	10 days	++ Blanching	1 hour	Slight blanchng
33	Same dose	24 hours	++++	4 days	+,+++ Necrosis and induration
34	Same dose	24 hours	++++	4 days	+++— Necrosis and induration

The uteri of guinea pigs both actively and passively sensitized gave typical reactions when the specific substance was added to the bath. The highest dilution employed gave a reaction in 1:100,000. Whether this was the minimal reacting dose cannot be stated.

Effect of Shock on Tuberculin Reaction

Simultaneously with the determination of these facts it was possible to observe the effect of anaphylactic desensitization on the allergic reaction in those animals which suffered acute sub-lethal shock. By injecting 0.1 cc. 1:10 dilution of O.T. intracutaneously before and after the shocking dose was given, any diminution in the intensity of the skin lesion could be nicely perceived. In Table V these comparisons are made.

From these data it becomes apparent that in guinea pigs made anaphylactically sensitive to the specific carbohydrate by intraperitoneal injections of killed tubercle bacilli, and showing at the same time inflammatory skin reactions to O.T., acute shock does not influence the intensity of the dermal response as indicated by subsequent tests with O.T. None of the guinea pigs passively sensitized with anti-Tb. rabbit serum showed a positive von Pirquet reaction when tested with O.T. This is an indication that the anaphylactic mechanism is totally distinct from the phenomenon of skin allergy in tuberculosis.

Whether or not the degree of anaphylactic sensitization runs parallel with the vigor of the cutaneous reaction cannot be judged from the results so far obtained.

DISCUSSION

Since these observations form merely the starting point for a more extended study of the problem of allergy in tuberculosis, no exhaustive discussion of the material presented will be undertaken at present, but will be deferred until experiments with the so-called nucleo-protein fraction in conjunction with the specific soluble substance both *in vivo* and *in vitro* have been completed. It is, however, pertinent at this point to inquire whether the instances of fatal shock given above were caused by the specific carbohydrate itself or a protein impurity, and then to ascertain whether actual anaphylactic shock has been produced according to the criteria laid down by Wells (6).

Since the specific polysaccharide contains 0.3 per cent nitrogen, it would seem theoretically impossible to exclude the participation of a protein in the reaction—but this is exceedingly unlikely if we consider the small amount of protein represented by the lethal dose of 2 mg. of carbohydrate. If all the nitrogen present be taken as included in

protein, the latter would weigh about 0.0375 mg. According to Wells (7), the minimum lethal dose of purified protein is about 1/20th or 1/10th of a milligram. The process of purification, however, as well as the failure to obtain any qualitative tests for protein make it highly improbable that the nitrogen present represents a whole protein or even any of its higher derivatives. We may thus conclude with reasonable certainty that the substance responsible for acute death is of the nature of a carbohydrate.

All but one of Wells' criteria for true anaphylaxis have been met in these experiments: (1) that the material be toxic for sensitized animals only; (2) that the symptoms produced be characteristic for the species of animal employed; (3) that passive transfer of sensitization be possible (this has been done only with rabbit serum, not with the homologous serum of the sensitized animal); (4) that capillary thrombosis or emboli be not the cause of death (this is excluded only by the number of animals suffering shock and the typical autopsy findings); (5) that desensitization take place after non-fatal shock (2 animals tested, neither reacted to 2 mg. of the specific soluble substance).

Tests for the prevention or amelioration of bronchial spasm by the use of atropin and adrenalin have not been done.

We can then be fairly assured that we have been dealing with true anaphylaxis produced by the specific carbohydrate of the tubercle bacillus.

CONCLUSIONS

1. Typical lethal anaphylactic shock has been produced in guinea pigs by means of the specific carbohydrate derived from a human strain of tubercle bacillus. The phenomenon has been observed in animals both actively and passively sensitized.

2. Typical contractions have been evoked in the isolated uterus of guinea pigs upon the addition of the specific carbohydrate to the bath.

3. True anaphylactic shock against the specific carbohydrate in guinea pigs sensitized with the tubercle bacillus does not prevent or diminish the cutaneous reaction to O. T. in those animals which recover.

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SENSITIVITY TO METHYLENE BLUE AND FINAL ACID-ITY OF NON-HEMOLYTIC STREPTOCOCCI

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The differentiation of hemolytic streptococci by means of differences in the final hydrogen ion concentration and in sensitivity to methylene blue has been presented in a preceding paper (1). By means of these reactions three groups were distinguishable: (1) human strains, defined by a final pH range of 5.0 and by failure to reduce methylene blue (1:5000) in milk; (2) bovine strains from the udders of cows, characterized by a final pH of 4.5 to 4.2 and by failure to reduce methylene blue; (3) saprophytic strains from dairy products, showing a final pH of 4.5 to 4.2 and the capacity to reduce the dye during growth in milk.

The present paper presents the results of the application of the same tests to a collection of different strains of non-hemolytic streptococci isolated from human infections, bovine mastitis and dairy products.

Description of Strains

During the investigation 55 strains of non-hemolytic streptococci were examined. Of these, 12 strains were isolated from individuals suffering from endocarditis and septicemia, 6 from the udders of cows showing mastitis and 37 from dairy products such as butter starters, sour milk, and cheese. On blood agar plates all produced methemoglobin both on the surface and in the depth of the medium with the exception of three from dairy products which caused no change in the blood pigment. None of the strains except three from human source fermented inulin. All strains were insoluble in bile. The origin and number of strains from each source are given in Table I.

Final Hydrogen Ion Concentrations

To determine whether the non-hemolytic streptococci could be divided into high and low acid groups as the hemolytic strains had

been, the final hydrogen ion concentration in dextrose broth was tested.

Fifty-four of the 55 strains tested showed a range of pH from 4.9 to 4.1. Only one of the strains from human source showed a low-acid-production (pH 5.2) within the range given for the hemolytic strains isolated from human infection. Five non-hemolytic strains from human source and two from mastitis in cows showed final hydrogen ion concentrations between the limits distinctive for human and bovine types of hemolytic streptococci (pH 5.0 to 4.5).

No distinction between strains from bovine and human sources could be demonstrated therefore, by means of differences in their final hydrogen ion concentrations.

TABLE I

Non-Hemolytic Streptococci

Source	Number of strains
Endocarditis: septicemia.	12
Bovine mastitis.	6
Sour milk, cream.	18
Butter starters.	10
Ice cream.	1
Cheese.	8
Total.	55

Reduction of Methylene Blue Milk

The capacity of the non-hemolytic streptococci to reduce methylene blue milk was tested by the method described in the preceding paper (1). Methylene blue was added to milk in final concentration of 1:5000, 1:10,000 and 1:20,000.

The results of the study of the reducing action of these strains are given in Table II.

Analysis of the data (Table II) shows that with the non-hemolytic streptococci as previously with the hemolytic variety, strains of saprophytic origin have a greater tendency to tolerate and to reduce high concentrations of methylene blue than have strains of parasitic (human or bovine) origin. This tendency is evidenced by the fact that 14 of the 18 strains from human and bovine sources failed to

reduce a 1:5000 concentration of the dye whereas 32 out of the 38 strains from saprophytic sources not only actively reduced but grew luxuriantly in the same concentration.

However the difference in sensitivity to methylene blue was not so pronounced and clean cut with the non-hemolytic streptococci as it was with the hemolytic strains (1); and although a number of different concentrations of the dye were tested, no satisfactory distinction could be obtained between the strains from human and bovine infections and the saprophytic strains from dairy products. When the concentration (1:20,000) was sufficiently low to permit reduction on the part of all the saprophytic strains, the dye was likewise tolerated by more than half of the strains from human sources and from bovine

TABLE II

Reduction of Methylene Blue by Non-Hemolytic Streptococci

Concentration methylene blue in milk	Strains isolated from					
	Human infections		Bovine mastitis		Dairy products	
	Not reducing	Reducing	Not reducing	Reducing	Not reducing	Reducing
1:20,000	5	7	1	5	0	38
1:10,000	7	5	2	4	1	37
1:5,000	11	1	3	3	6	32

udders. Increasing the concentration of the dye was of no advantage: when the concentration (1:5000) was sufficiently high to prevent reduction on the part of most of the human and bovine strains, about one-fifth of the saprophytic strains proved unable to tolerate the dye.

Hence, although the total number of strains studied was not large, the results definitely show that with non-hemolytic streptococci differences in sensitivity to methylene blue fail to give a distinction of practical value between strains from pathogenic and those from saprophytic sources.

DISCUSSION

Previous work (1) has shown that hemolytic streptococci may be divided into three groups on the basis of differences in the final hydrogen ion concentration and in sensitivity to methylene blue. The data in this paper shows that the non-hemolytic streptococci cannot

be similarly differentiated by these reactions. The cultures from human source with one exception, attain a final hydrogen ion concentration comparable to that of the strains isolated from milk and from cows udders. These results are in agreement with the observations of Ayers, Johnson and Davis (2) who found that 95% of the non-hemolytic streptococci from feces and udders of cows had the same range of final hydrogen ion concentration (pH 5.0–4.5) as had strains from human infection. Moreover, differences in sensitivity to methylene blue of the non-hemolytic strains of saprophytic and parasitic origin are not sufficiently marked to be of differential value. It has been observed by many investigators that the saprophytic group of lactic acid streptococci (*Strep. lacticus*) which predominate in many dairy products, are in general characterized by a marked capacity to reduce dyes. The reducing capacity of strains from saprophytic sources previously reported (1) for hemolytic streptococci is also evident among the non-hemolytic streptococci. However, the lack of correlation between acid production and sensitivity to methylene blue among the non-hemolytic streptococci is in striking contrast to the definite relationship found within the hemolytic group. In this connection it is interesting to note that Ayers and Rupp (3), by means of the hydrolysis of sodium hippurate were able to distinguish hemolytic streptococci isolated from the udders of cows from those derived from human sources. But, when the same test was applied to the non-hemolytic streptococci, they found a number of strains from the udder which did not produce the hydrolysis typical of the hemolytic variety from this source.

This lack of uniformity in the biochemical reactions of the non-hemolytic streptococci is also apparent in the lack of specific relationships demonstrated by immunological methods. Kinsella and Swift (4) found that the non-hemolytic streptococci are antigenically heterologous. On the other hand the hemolytic group from human sources has been shown by several investigators (5, 6) to consist of types defined by a considerable degree of immunological specificity.

The results of the present investigation support the view that in their biochemical reactions as in their serological behavior the non-hemolytic streptococci are a more heterologous group than are the hemolytic streptococci.

SUMMARY

The results of the present study may be briefly summarized as follows. Acid production and sensitivity to methylene blue of 55 strains of methemoglobin-forming streptococci from various sources were studied to determine whether it is possible on the basis of these reactions to differentiate strains of human, bovine, and dairy origin.

Twelve strains were isolated from human infections, 6 from bovine mastitis, and 37 from dairy products. The final hydrogen ion concentrations of all cultures in dextrose broth regardless of the sources from which the strains originated, covered the range pH 5.2 to 4.1. There appeared, therefore, to be no correlation between the amount of acid produced by a given strain and the source from which it was originally derived. Low acid production, a characteristic of hemolytic streptococci of the human type, was not a distinguishing mark of the green producing organisms from human infection, and high acid production was not limited to strains from bovine and dairy sources.

Differences between saprophytic and parasitic strains in their sensitivity to methylene blue in milk were not so sharply defined as were those previously demonstrated for the hemolytic streptococci. Of 18 strains of human and bovine infections, 14 showed neither reduction nor growth in the presence of a 1.5000 concentration of methylene blue. Of the 38 strains from dairy products 32 reduced the dye and grew in this concentration.

The results with the non-hemolytic streptococci indicate that the saprophytic strains have a greater tolerance for the dye than have the strains of parasitic origin.

The significance of these results in the differentiation of streptococci of human, bovine and dairy origin is discussed.

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STUDIES ON YELLOW FEVER IN SOUTH AMERICA

IV. Transmission Experiments with Aëdes aegypti

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During the first few months following the establishment of Brazilian
strains of yellow fever virus in this laboratory a great many attempts
were made to transmit the infection with *Aëdes (Stegomyia) aegypti*,
which had previously engorged on rhesus monkeys in the early febrile
stage of the disease. Recently it has been found more practicable to
maintain the strains by transfer of fresh or dried blood and tissues
containing virus.

Successful Transmission by Mosquitoes

In Table I are listed eighteen fatal infections produced in monkeys
by bites or injection of mosquitoes, or by a combination of the two
procedures. Fifteen of these infections were due to Brazilian strains
and three to the Asibi, or African, strain. In Table II the fifteen fatal
infections are briefly compared with seventeen non-fatal infections due
to the Brazilian strains.

The average maximum temperature was slightly higher in fatal
infections and the incubation period was usually shorter. However,
among non-fatal cases the incubation period varied a great deal, i.e.,
from less than twenty-four hours to fifteen days. In three instances
fever appeared on the day following the infective feeding. These
early fevers were due twice to the F. W. strain and once to the S. R.
strain of virus. Stokes, Bauer and Hudson (1) give examples of mon-
keys showing fever the day after they were fed upon by mosquitoes.
The first rise in temperature to 104°F. occurred once on the ninth day,
once on the tenth day, once on the fourteenth day and once on the
fifteenth day. Three of these four cases with rather prolonged incu-

TABLE I

Fatal Infections from Mosquito Transmission

Rhesus No.	Strains of virus	Mosquito Lot No.	Fed or injected	Day of feeding or injection	Temperatures recorded A.M. and P.M. by days after infection									
					1	2	3	4	5	6	7	8	9	10
A1	B. B.	22	Fed	—	102.4	102.3	103.7	102.9	104.5	105.7	96.6			
				102.2	102.6	102.5	103.0	103.2	104.7	104.9	D			
A2	F. W.	11; 12; 13; 15	Fed	102.8	102.0	102.4	103.2	103.4	103.7	105.0	103.9	D		
				102.9	103.0	103.5	102.9	103.9	104.2	105.5	102.3	—		
A3	B. B.	19; 25; 26	Injected	—	103.9	103.9	102.7	102.5	103.9	103.7	104.6	103.9		
				103.5	104.3	104.0	102.8	102.8	104.9	104.5	104.9	102.0		
A4	B. B.	34	Fed	—	103.2	102.8	105.6	104.7	104.8	D				
				103.4	103.6	103.3	105.4	105.0	103.6	—				
A5	B. B.	44	Fed	—	103.1	102.7	102.9	104.9	104.9	106.0	104.0	D		
				102.9	103.4	103.4	103.6	104.7	105.4	105.5	101.9	—		
A6	Asibi*	79	Fed	—	103.2	103.8	105.0	104.0	D					
				103.0	103.6	103.9	105.4	103.8	—					
A7	F. W.	77; 78	Fed	—	102.8	103.4	104.1	105.0	104.6	102.2	D			
				102.0	103.0	103.3	105.2	105.8	104.1	102.0	—			
A8	B. B.	104	Fed	—	103.6	103.7	104.7	104.2	103.9	D				
				103.1	103.8	103.4	104.9	104.8	103.7	—				
A9	S. R.	98	Fed	—	102.7	103.3	103.8	104.0	104.0	104.7	104.0	104.1	D	
				102.8	103.0	103.7	103.9	104.0	103.9	104.5	103.5	S	—	
A10	Asibi*	93	Fed	—	102.9	103.2	105.4	103.7	D					
				103.6	103.1	103.4	104.7	104.7	—					
A11	B. B.	70; 76; 66	Fed and injected	—	102.8	103.2	103.5	103.3	105.6	104.0				
				102.6	103.5	103.7	103.4	104.3	102.9	S				

No.	Strain		Treatment											
A12	S.R.	72; 92	Fed	—	102.0	102.7	103.0	103.4	103.5	103.7	103.9	102.9	D	
				102.3	102.2	102.8	103.2	103.6	103.4	104.5	101.1	103.8	—	
A13	Asibi*	81	Fed	—	102.9	103.6	103.4	105.2	104.7	105.2	104.5			
				102.8	103.4	103.8	103.9	104.6	104.9	105.4	S			
A14	B. B.	99	Fed	—	103.8	103.4	103.2	104.6	104.7	104.0				
				104.3	103.5	103.6	102.9	104.2	105.8	S				
A15	B. B.	119; 124; 99	Fed and injected	—	102.9	102.5	104.3	104.0	105.9	104.8	D			
				102.2	103.6	102.3	104.9	106.4	106.0	103.6	—			
A16	S. R.	126; 133; 98	Fed and injected	—	102.7	101.9	100.0	104.2	103.8	104.8	104.2	103.9	103.5	100.0
				102.9	102.4	101.4	102.6	104.0	103.9	104.6	104.0	103.7	101.7	S
A17	S.R.	152; 153	Fed	—	103.0	103.6	104.0	103.9	104.9	104.6	S			
				102.7	103.4	103.5	103.8	105.0	104.8	104.8	—			
A18	N. C. D.	164; 171	Fed	—	102.7	103.0	105.2	104.8	D					
				102.9	102.9	103.2	105.0	104.0	—					

D—spontaneous death from yellow fever. S—sacrificed when *in extremis*. *The Asibi strain is of African origin.

bation time were proved to be yellow fever by successful transfer of blood or by infecting mosquitoes at the time of the febrile access. It is presumed that the fourth case of fever (fourteen days' incubation) was also specific because the mosquitoes used had previously transmitted the infection and because suggestive lesions were found at autopsy a few days after the attack. Marchoux, Salimbeni and Simond (2) record an experiment in which a human subject showed fever for the first time on the thirteenth day following inoculation.

Production of Immunity without Symptoms of Disease

From October, 1928, to February, 1929, forty-seven transmission experiments were attempted with lots of mosquitoes which were

TABLE II

Comparison of Fatal and Non-Fatal Infections.   Brazilian Strains Only

	Total cases	Average incubation period*	Average febrile period Days**	Average maximum temperature	Average number days from beginning experiment to death of monkey
Fatal.....................	15	3.6	3.0	105.4	7.1
Non-fatal.................	17	5.3	3.5	104.9	—

*Days from application of mosquitoes to first fever.
** A temperature of 104.0 has been accepted as indicating fever.

thought to be infected. Most of these lots were originally fed on monkeys on the first day of fever, a few were fed on the second day. In many instances two or more batches were used on the same animal. From this total are excluded experiments which utilized mosquitoes fed by Dr. Bauer on infected monkeys in Rio de Janeiro, mosquitoes fed on doubtfully infected rhesus monkeys in Bahia, and mosquitoes fed on native Brazilian monkeys. Only twenty-three, or 48.9 per cent, of the experiments were successful.

During this same period forty-nine direct transfers of blood were made from monkeys with fever to normal monkeys. Three times the inoculation was made on the second day of fever and three times on the third day; in the other forty-three instances it was made on the first day. In nine of the latter experiments, however, another transfer

was made on the second or third day from the same donor. The series does not include the inoculation of fresh bloods from multiple sources, or the inoculation of dried blood or of tissues. Forty, or 81.6 per cent, of the experiments were considered positive. Nearly all of these transmissions were carried out with Brazilian strains of relatively low virulence. Four of the nine negative results were with the F. W. strain of virus which has always acted erratically.

There was a considerable discrepancy between the number of positive mosquito transmissions and the number expected, as judged by direct inoculation of blood. The animals which failed to react to mosquito bites were tested for immunity by the direct inoculation of blood or tissues containing virus. In every case where the mosquitoes were fed in the first instance on an animal known to have been infected, the monkeys bitten subsequently showed some degree of immunity. This refers only to transmission experiments with the "yellow fever mosquito," *Aëdes aegypti;* other species of mosquitoes will be considered in later publications.

Table III summarizes the testing of thirteen animals which showed no fever from mosquito bites. In some cases the immunity test caused a febrile reaction. *M. rhesus* No. B 15 was fed upon by mosquitoes which had previously engorged on a *Cebus* monkey; the temperature following the immunity test rose once to 104°F. Besides the cases given in Table III, the records show fifteen other experiments with failure to produce fever in which the first host for the mosquitoes was a rhesus monkey, also a number involving Brazilian monkeys. Unfortunately, in many of these experiments the test virus was weak and results are correspondingly less convincing.

To the list in Table III might well be added an experiment on *M. rhesus* No. D 1. This monkey had been fed upon by certain mosquitoes and inoculated with others, all from batches infected with F. W. strain of virus. On the initial day the temperature was 103.9°; on the following day it was 103.8°; on the fourth and fifth days it rose to 104° but it never went above this. The rise was slight, but possibly significant. On the sixteenth day the animal received a test dose of virus. Similar doses of this virus killed the control and eight other animals. On the day of the test inoculation, No. D 1 had a temperature of 103.4°. The highest temperature noted during the observation period of the immunity test was 103.7°, on the eighth day. There are other records similar to that of No. D 1 which have been excluded from consideration because of the empirical rule that a temperature of 104°F. indicates fever.

TABLE III

Monkeys Showing No Fever from Mosquitoes; Subsequently Immune to Test Inoculation

		Experiment with mosquitoes			Maximum temp.				Test virus		Immunity test — Maximum temp.			Control				
Monkey No.	Mosquito Lot No.	Strain of virus	Fed or injected	Initial temperature	Degrees	Day of expt.	Days under observation	Days elapsed between mosquito expt., and immunity test	Strain	Source Monkey No.	Degrees	Day of expt.	Days under observation	Incubation period (days)	Maximum temp.	Duration of fever (days)	Death or recovery	Days elapsed to death
B1	7	F. W.	Fed	103.0	103.4	4	20	56	B. B.	C1	103.7	4	20					
B2	33	B. B.	Fed	102.8	103.7	15	17	5	B. B.	C1	103.7	1; 2	20					
B3	22; 24; 27	B. B.	Injected	103.0	103.9	1	18	none	B. B.	C1	103.7	3	20					
B4	36; 37	B. B.	Fed	102.4	103.0	9	15	none	B. B.	C1	102.2	3	13*					
B5									B. B.	C1				1.5	105.4	1.5	D	3.5
B6	62; 63; 65	B. B.	Fed	103.3	103.8	1; 6	19	12	Asibi	C2	103.8	6	20	1.5	105.7	2	D	4
37									Asibi	C2								
	32; 43	B. B.	Injected	103.2	103.7	12	14		B. B.	C3	103.1	12	19					
	42	F. W.	Injected	103.0	103.4	7	9		B. B.	C3	103.8	3; 5	19					
	47; 51	F. W.	Fed	102.0	103.4	7	8		B. B.	C3	103.6	5	19					
	49; 56	B. B.	Injected	102.8	103.4	6	8		B. B.	C3	103.7	5; 11	19					
	48; 52; 82	B. B.	Fed	102.2	103.7	9; 12	15	15	B. B.	C4	103.7	9	4		105.7	7	R	
									B. B.	C4			3		105.4	3	R	

V	B15	97	Asibi	Fed	102.0	103.7	5	16	none	Asibi	C5; C6; C7	104.0	8	19	5	105.0	2	D	7.5
	B16									Asibi	C5; C6; C7								
VI	B17	91; 143	F. W.	Fed and	102.0	103.5	13	16	none	Asibi	C8**	103.6	1	21	6	105.6	3	R	
	B18			injected						Asibi	C8**								
VII	B19	139; 141	S. R.	Fed	102.9	103.7	4	17	none	Asibi	C9; C10	103.7	3	19	3	105.6	1.5	D	5
	B20									Asibi	C9; C10								

* *M. rhesus* No. B 4 died from enteritis during the observation period following immunity test.

** Virus from *M. rhesus* No. C 8 killed six animals, but the control survived.

Certain lots of mosquitoes have produced fatal yellow fever in one monkey and no febrile reaction in a second. For example, mosquito batch No. 22, infected from rhesus No. D 2, later caused the death of rhesus No. D 3, but no fever in No. D 4. Without the isolation of individual mosquitoes it is impossible to say whether all of the insects which fed on the first animal also fed on the second, although the majority of the batch engorged on each occasion.

DISCUSSION

The foregoing observations show that batches of mosquitoes, even if certainly known to carry the yellow fever virus, do not necessarily cause frank manifestations of disease in every animal upon which they feed. Undoubtedly, some of the monkeys which fail to react are naturally immune, but our experience with direct transfers of blood and tissues proves that such animals account for only a part of the total failure in mosquito transmission.

It has been shown that the animals which do not show febrile reactions following the bites of infected mosquitoes are, nevertheless, resistant to further inoculations. It would seem that the animals are immunized by subinfective doses of virus, with or without a slight rise in temperature. Immunization without obvious yellow fever conforms with Finlay's (3) theories of protection and harmonizes with present views of sub-clinical immunization in other infections. In our experiments every lot of mosquitoes had presumably reached the infective stage before biting was permitted.

We have observed lots of mosquitoes to become infective when the fever in the host was slight and of short duration. Fatal infections arose from the bites of mosquitoes fed on animals with severe, although not necessarily fatal infections; and mild reactions or immunity without manifest disease followed the bites of mosquitoes fed on animals with either mild or severe infections.

If it be permissible to use these experiments performed upon monkeys to help elucidate certain factors in the epidemiology of yellow fever in man, we have a possible explanation for (1) widespread immunity or partial immunity in a native population where frank cases of yellow fever are rare, and (2) the presence of recognized sporadic cases where preceding cases of the disease cannot be traced. It is

suggested that in nature infected mosquitoes may establish immunity, or renew immunity, in several individuals for every one in which a frank attack of yellow fever is produced.

A history of the strains of virus mentioned in this paper has been given in a previous publication (4).

SUMMARY

1. Batches of *A ëdes (Stegomyia) aegypti* which had fed on monkeys in the early febrile stage of yellow fever and which has subsequently passed the usually accepted extrinsic incubation period for the virus, failed to transmit the disease to normal monkeys in approximately fifty per cent of the experiments. During the same time over eighty per cent of blood transfers were successful.

2. The monkeys which failed to show fever following mosquito bites later proved resistant to the inoculation of blood or tissues containing virus.

3. The incubation, or afebrile, period in monkeys following the bites of infected mosquitoes varied from less than twenty-four hours to fifteen days. It averaged somewhat longer in non-fatal than in fatal infections.

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STUDIES ON YELLOW FEVER IN SOUTH AMERICA

V. Transmission Experiments with Certain Species of Culex and Aëdes

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Bauer (1) has reported the transmission of yellow fever virus by several species of African mosquitoes, all but one of which belong to the genus Aëdes. The Western Hemisphere contains no close relatives of the "yellow fever mosquito," *Aëdes (Stegomyia) aegypti,* but we have recently conducted experiments with a number of species belonging to other groups, which are common in the American tropics, in order to determine whether any of these were capable of transmitting the virus.

The common house mosquito of the tropics and subtropics, *Culex quinquefasciatus (C. fatigans),* has engaged the attention of yellow fever workers as a possible transmitter, but no positive evidence has ever been adduced against it. Both The American Yellow Fever Commission (2) in Cuba and the French Commission (3) in Rio de Janeiro obtained negative results in experiments with this species. The French Commission also carried out one experiment with *Aëdes (Ochlerotatus) scapularis* (called by them *Culex confirmatus*) and one with a *Culex* of uncertain species, both without success. The experiments of these two commissions were all upon human volunteers.

RESULTS OF EXPERIMENTS

Utilizing as an experimental animal *Macacus rhesus,* we have carried out a number of feeding and inoculation experiments with South American mosquitoes. The accompanying table summarizes the results with *Culex quinquefasciatus, Aëdes (Ochlerotatus) scapularis, Aëdes (Ochlerotatus) serratus* and *Aëdes (Taeniorhynchus) taeniorhynchus.*

Summary of Transmission Experiments

Animal No.	Mosquito Lot No.	Experiments with mosquitoes								Immunity test					Control to immunity test				
		Species of mosquito	Fed or injected	Number of mosquitoes	Virus strain	Fever	Duration of fever (days)	Maximum temperature	Death or recovery	Test strain	Fever	Duration of fever (days)	Maximum temperature	Death or recovery	Animal No.	Fever	Duration of fever (days)	Maximum temperature	Death or recovery
A1	106	C. quinquefasciatus	Fed	5	Asibi	−	—	103.5	R	Asibi	+	3	104.7	R	B1	+	2	105.0	D
A2	106	C. quinquefasciatus	Injected	13	Asibi	+	4	104.1	R	Asibi	+	4	104.7	R	B2	+	2	105.1	D
A3	132 & 146	C. quinquefasciatus	Injected	4	1 B. B. / 3 Asibi	+	4	104.6	R	Asibi	+	4	105.8	R	B3**	+	3	105.6	R
A4	166	C. quinquefasciatus	Fed	4	Asibi	−	—	103.4	R	Asibi	+	3	105.0	D	B4*	+	3	106.1	D
A5	165	C. quinquefasciatus	Injected	9	Asibi	−	—	103.5	R	Asibi	+	2	105.9	D	B4*	+	3	106.1	D
A6	193 & 194	C. quinquefasciatus	Injected	16	Asibi	−	—	103.6	R	Asibi	+	7	104.5	R	B5***	+	2	104.4	D
A7	83	Aëdes scapularis	Fed	20	B. B.	−	—	103.8	R	Asibi	−	—	103.6	R	B6	+	9	105.4	R
A8	83	Aëdes scapularis	Injected	62	B. B.	+	3	106.4	D										
A9	129	Aëdes scapularis	Fed	10	B. B.	+	—	103.5	R	Asibi	−	—	103.6	R	B7****	−	—	103.6	D
A10	129	Aëdes scapularis	Fed	1	B. B.	−	—	106.0	R										
A11	130	Aëdes scapularis	Injected	4	B. B.	+	1	105.2	D										
A12	172	Aëdes scapularis	Fed	12	Asibi	+	2	103.5	R	Asibi	+	2	104.5	R	B4*	+	3	106.1	D
A13	174	Aëdes serratus	Fed	2	Asibi	−	—	103.5	R	Asibi	+	3	105.2	D	B4*	+	3	106.1	D
A14	174	Aëdes serratus	Injected	8	Asibi	+	3	105.4	D										
A15	183	Aëdes taeniorhynchus	Fed	13	Asibi	+	2	104.2	R	Asibi	+	3	105.8	D	B8	+	3	105.8	D
A16	192	Aëdes taeniorhynchus	Fed	7	Asibi	−	—	103.6	R	Asibi	+	7	104.7	R	B9	+	3	104.4	D
A17	183 & 192	Aëdes taeniorhynchus	Injected	25	Asibi	+	3	104.2	R	Asibi	−	—	103.6	R	B5***	+	2	104.4	D

*M. rhesus No. B 4 served as control in four of the immunity tests. The same dosage of virus killed eight other animals.

**The same dosage of virus killed six animals, but the control, No. B 3, survived. No. B 3 had previously been fed upon by normal Aëdes aegypti.

***M. rhesus No. B 5 served as control in two of the immunity tests.

****Control, No. B 7, died from yellow fever on the fourth day, although he had shown no fever at the hours of taking temperature.

No certain transmission was secured either by the bites or by the inoculation of Culex, although two of the experimental monkeys showed slight fever.   It would appear, however, that in three of the experiments (rhesus Nos. A 1, A 2 and A 6) a low grade of immunity was produced.   This was more noticeable in the cases of rhesus Nos. A 1 and A 2, in which Culex batch No. 106 was used.   One can only conjecture whether attenuated virus actually survived in this species.

With *Aëdes scapularis* the results were different.   *M. rhesus* No. A 8 died after being bitten by, and inoculated with, the ground up bodies of this species.

The lot used was a combination of lots fed on four different infected rhesus monkeys between December 10 and 14, 1928.   On January 2 and 3, 1929, the mosquitoes were allowed to feed on rhesus No. A 7.   On January 7 six of the batch engorged on rhesus No. A 8.   On January 9 the sixty-two remaining mosquitoes of the lot were ground up in salt solution and inoculated subcutaneously into the same animal.   On January 13 the monkey's temperature rose to 105.0°F. and continued high for three days; on January 16 the animal died and the gross and microscopic lesions were typical of yellow fever.

Bites of *scapularis* lot No. 130 were sufficient to produce fatal yellow fever in rhesus No. A 11.   The lot received its infective blood meal from rhesus No. B 10 on February 14, 1929.   On February 27 about twelve of the mosquitoes engorged on rhesus No. A 11.   On the fourth day following, March 3, the animal had a temperature of 105.2°F.   On March 5, the temperature dropped to 98° and the monkey was killed when moribund.   The gross and microscopic lesions were typical of yellow fever.

M. rhesus No. A 10 had a definite febrile attack following the inoculation of *scapularis* of batch No. 129.

Animals Nos. A 7, A 9 and A 12 developed a high degree of immunity from the bites of *Aëdes scapularis*, without having shown any preceding fever.   Such immunity without manifest disease has been shown to follow frequently the bites of infected *Aëdes aegypti* (4).

Only one lot of *Aëdes serratus* was used.   Eight of these mosquitoes fed upon rhesus No. A 13 after an extrinsic incubation period of twenty-two days.   No fever resulted and the animal later succumbed to the immunity test.   The inoculation of thirteen of this lot on the thirty-first day after the infective blood meal produced a fatal attack of yellow fever in rhesus No. A 14.   It is evident that in this particular lot the virus did not enter the salivary glands, although it remained alive at some place in the body of the insects.

Two lots of *Aëdes taeniorhynchus* Nos. 183 and 192 were used. One of the two animals fed upon by these insects survived the immunity test. Probably virus remained alive in the body of this species of mosquito also.

Twenty-five *taeniorhynchus* were inoculated subcutaneously into rhesus No. A 17 on June 3, 1929. Twenty-four days had elapsed since the infective meal of batch No. 183, and twenty days since that of batch No. 192. At the time of inoculation the temperature of rhesus No. A 17 was 103.4°; on June 4 it rose to 104°; on June 5 it was 104.2°; and on June 6, 104° again; on June 7 it dropped by crisis to 101.8°. Thereafter it ranged between 102.6° to 103.6.° The immunity test caused no reaction; the highest temperature during the observation period was 103.5°. The control, rhesus No. B 5, which had been inoculated with the same virus as that used in the immunity test, had a typical attack of yellow fever and was killed on the fifth day when moribund. Autopsy findings were typical of the disease.

Lots of *Aëdes (Ochlerotatus) hastatus* were on several occasions given an opportunity to feed, but none were ever observed to take blood.

Habits of the Species of Mosquitoes Used for Experimentation

Culex quinquefasciatus breeds in domestic and peridomestic locations, usually in artificial receptacles, and by preference in water containing much decomposing organic material. The females feed only at night, but during the daytime adults of both sexes may frequently be found resting in bedrooms, closets, toilets and lavatories. In spite of the prevalence of the species and the ease with which it is reared, we were able to use only a few specimens in each experiment because the females showed no great avidity for monkey blood. Our method was to anesthetize the monkeys with amytal, place them in the mosquito cages at about five o'clock in the afternoon and leave them for three to four hours exposed to mosquito bites. It is quite possible that if the animals had been left with the mosquitoes all night, better results might have been secured.

Aëdes scapularis is widely distributed from the West Indies through Columbia, Venezuela, the Guianas, Brazil, Bolivia and Northern Argentina. It breeds in temporary rainpools both in rural areas and in urban districts. In shaded localities it will attack man or animal at any hour of the day and will even pursue its victim into the sunlight. It attacks in greatest numbers at dusk. Although not truly domestic it freely invades yards and gardens and will occasionally enter houses to secure blood. Upon rare occasions it has been seen resting indoors during the daytime. It was found to feed readily on monkeys, but the adults did not survive as long in confinement as did specimens of *Aëdes aegypti*.

Aëdes serratus is found in Mexico, Central America, northern South America and

Brazil (5). It breeds in temporary rainpools. In this section of the country we have seen it only in rural and sylvan localities; it appears to be much less ubiquitous than *scapularis*. In nature it feeds by preference in the 'evening, but in the laboratory it engorged on monkeys at various hours of the day.

Specimens which resemble quite closely the Central American *Aëdes hastatus* were bred out from rainpools occurring in the same neighborhood where *serratus* was found. The adults appear smaller and more delicate than either *scapularis* or *serratus*. In spite of repeated opportunities to feed during daylight hours they were never observed to take blood in the laboratory. It is possible that in nature they feed at night or upon some particular host only. We have not found them in collections made when using animal bait.

Aëdes taeniorhynchus is the prevalent salt marsh mosquito of the South Atlantic and Gulf Coast states of the United States of America. It extends south through Mexico and Central America to Peru on the west coast and to Brazil on the east coast of South America. Dyar (5) in his recent book does not include Brazil in the distribution of the species. Peryassu (6) records it, under the name *Culicelsa taeniorhynchus* from Para, state of Rio de Janeiro, and the Federal District in Brazil. The French Commission (3) while in Rio de Janeiro kept specimens of "*Culex taeniorhynchus*"in captivity to observe the length of life and the feeding and egg-laying habits, but carried out no transmission experiments with them. During the month of April, 1929, the species was found breeding in crab-holes along the edge of the bay and also in rock-pools along the shore, in the city of Bahia. The crab-holes were above the level of high tide and appeared to contain only fresh water, probably largely rain-water. In the same month we received specimens of *taeniorhynchus* from Aracaju in the state of Sergipe and heard later that the species had been found in Alagoas. This mosquito is a vicious biter but fortunately has a rather limited breeding season.

DISCUSSION

Our experiments show that *Aëdes scapularis* is capable of transmitting yellow fever by its bite. Whether it ever becomes a factor in the dissemination of the disease is not known. Its feeding habits do not preclude this possibility, because many yellow fever patients are not bedridden at first and may expose themselves to the bites of mosquitoes outdoors, and also because the insect at times enters houses in search of blood.

The tests have shown that yellow fever virus may survive for some time in the bodies of *Aëdes serratus*, and probably also in *Aëdes taeniorhynchus*; but whether these species ever become capable of transmitting the virus by their bites is not yet known. By mashing an infected mosquito on the skin a person might conceivably become

infected. The biting habits of *Aëdes taeniorhynchus* are such as to bring it under suspicion during the season of maximum prevalence if yellow fever is present in the vicinity.

There is no evidence that *Culex quinquefasciatus* is able to transmit the disease in a recognizable form. However, in certain animals an apparent immunity to yellow fever was found to have developed subsequent to the bites of this species. We do not wish to over-emphasize this point, because the experiments were not as clear-cut as might be desired.

SUMMARY AND CONCLUSIONS

1. Yellow fever virus has been transmitted from monkey to monkey both by the bites of *Aëdes (Ochlerotatus) scapularis* which had fed upon monkeys infected with yellow fever and by the injection of the ground up bodies of such mosquitoes.

2. A fatal infection has been obtained by the injection of the ground up bodies of *Aëdes (Ochlerotatus) serratus*, which had previously fed on an infected monkey, and a mild infection has been secured by the similar injection of *Aëdes (Taeniorhynchus) taeniorhynchus*.

3. No definite infection has been secured either by the bites or by the injection of *Culex quinquefasciatus (C. fatigans)*. However, some of the experimental animals bitten by this species have been relatively immune following inoculations of blood or tissues containing virus.

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A QUANTITATIVE STUDY OF THE PRECIPITIN REACTION BETWEEN TYPE III PNEUMOCOCCUS POLYSACCHA-RIDE AND PURIFIED HOMOLOGOUS ANTIBODY*

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Of all the reactions of immunity the precipitin test is perhaps the most dramatic and striking. While other immune reactions are more delicate, the precipitin test is among the most specific and least subject to errors and technical difficulties. Attempts at its quantitative interpretation and explanation (1, 2) have been hampered either by the difficulty of finding suitable analytical methods† or by the failure to separate the reacting substances from closely related, non-specific materials with which they are normally associated.

With the aid of recent work it has been found possible to avoid these difficulties to some extent. The isolation of bacterial polysaccharides which precipitate antisera specifically (3) and possess the properties of haptens (4) has not only afforded one of the components of a precipitin reaction in a state of comparative purity, but has greatly simplified the analytical problem. Since many of these polysaccharides contain no nitrogen, and antibodies presumably are nitrogenous, the latter may be determined in the presence of any amount of the specific carbohydrate. Moreover, Felton's method for the separation of pneumococcus antibodies from horse serum (5) not only permits the isolation of a high proportion of the precipitin, freed from at least 90 per cent of the serum proteins and much of the serum lipoid, but is also applicable on a sufficiently large scale to furnish the amounts of antibody solution needed to make quantitative work possible. It is realized that antibody solutions of this type do not contain pure

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† Ingeniously solved by Wu over much of the reaction range.

antibodies—indeed, only 40 to 50 per cent of the nitrogen is specifically precipitable—but since so small a proportion of the original serum protein remains with the antibody a far-reaching purification actually has been effected.   It should thus be possible with the aid of antibodies purified by Felton's method to obtain data of a preliminary character which should point toward the mechanism of the reaction.   The present paper is concerned with such data obtained in a quantitative study of the precipitin reaction between the soluble specific substance of Type III pneumococcus and Type III pneumococcus antibody solution.

EXPERIMENTAL

1. Materials and Methods.—a. Solutions of Soluble Specific Substance, Type III Pneumococcus.—The soluble specific substance of Type III pneumococcus (6)* used was kindly supplied by Drs. O. T. Avery and W. F. Goebel of The Rockefeller Institute for Medical Research.   It was ash-free, contained 0.04 per cent of nitrogen, and showed $[\alpha]_D = -32°$.   A weighed amount of anhydrous substance was suspended in 0.9 per cent saline, dissolved with the aid of 0.1 normal sodium hydroxide, and the solution was diluted with saline, adjusted to pH 7.6 and made up to volume with saline to yield a 1 per cent solution.   This was sterilized in the autoclave and used as a stock solution for making up other dilutions.   These were prepared with sterile saline under aseptic precautions, and were kept in the icebox.

b. Type III Pneumococcus Antibody Solution.—The antibody solutions used were prepared essentially according to Felton's procedure (*loc. cit.*) from Type III antipneumococcus horse serum containing no preservative and supplied by the New York State Department of Health through the courtesy of Dr. A. B. Wadsworth and Dr. Mary B. Kirkbride.   100 to 200 cc. of serum were stirred slowly into 20 volumes of ice-cold water containing 9.5 cc. of molar potassium dihydrogen phosphate and 0.5 cc. of molar dipotassium hydrogen phosphate per liter.   The final pH varied from 5.6 to 6.3.   After standing over night in the cold the supernatant was decanted and the precipitate was centrifuged off in the cold† and dissolved in a volume of chilled 0.9 per cent saline equal to that of the serum taken. 0.1 normal hydrochloric acid was then added until a precipitate no longer formed on dilution of a test portion with two volumes of water, after which 0.1 normal sodium hydroxide solution was added until a slight precipitate again formed on dilution.   In general, 5 cc. of acid and 1.5 cc. of alkali per 100 cc. of serum were satis-

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* Subsequently referred to as SSS III.

† An International Equipment Co. refrigerating centrifuge with external brine coils was used throughout the work.

factory, although as Felton emphasizes, different lots vary and no absolutely definite procedure can be given. In the present work the process of purification was followed either by testing the agglutinating power of the fractions against a heat-killed Type III pneumococcus vaccine, or by the precipitin reaction, or by both methods. After addition of the alkali the opalescent solution was diluted with 2 volumes of water and centrifuged in the cold. The almost inactive precipitate was discarded and the supernatant poured into 6.7 volumes of the chilled buffer solution previously used, (equivalent to 20 times the volume of saline employed), also adding enough 0.1 normal sodium hydroxide to neutralize the remaining acid. The resulting precipitate was collected and dissolved in a volume of 0.9 per cent saline equal to that of the serum taken, and the pH was adjusted to 7.6. The solution was sterilized by passage through a Berkefeld N grade filter which previously had been washed with saline containing a drop of normal sodium hydroxide, followed by saline alone.

Antibody solutions prepared in this way were found to be rather unstable under the usual conditions of the precipitin test, and it therefore was necessary to subject them to a preliminary "ageing" treatment in order that control solutions might be relied upon to remain clear. This consisted in immersing the solution in a water bath at 37° for 2 hours, letting stand in the ice-box over night, centrifuging off the precipitate which usually formed, readjusting the pH if necessary, and filtering through a Berkefeld candle prepared as above. This treatment was repeated as many times as necessary, but the solutions usually remained clear after the second incubation at 37°. Much time was lost and very inconstant results were obtained until "ageing" was resorted to.*

The relative antibody content of the resulting solutions was estimated by determining the agglutination titer against a single heat-killed Type III pneumococcus suspension.

It will be seen from Table I that the agglutination titer and the maximum amount of protein precipitable by the type III polysaccharide ([total N—N in supernatant] × 6.25) are approximately proportional. The latter may therefore be taken as a more definite, though not necessarily more accurate, measure of the actual antibody content of the solutions.

It is also evident that the antibody in all of these solutions has been purified to approximately the same extent, since the ratios of protein precipitable by SSS III to total protein are not very different.

c. Analytical Procedure.—Sterile calibrated pipettes were used for all measurements and the greatest care was taken to keep the solutions sterile throughout the experiments.

5 cc. portions of the "aged" antibody solution were pipetted into 15 cc. Pyrex centrifuge tubes. Solutions of SSS III of the required concentrations were added and the volume was made up to 10 cc. with 0.9 per cent salt solution. A blank containing 5 cc. of antibody and 5 cc. of saline was set up at the same time. The contents of the tubes were thoroughly mixed as quickly as possible. The mixtures were then incubated for 2 hours at 37°C. and allowed to stand over night in the ice-box. The precipitate was centrifuged off in the cold and duplicate 2 cc. samples of the supernatant were analyzed for nitrogen, using the Pregl micro-Kieldahl method with N/70 acid and alkali. The amount of nitrogen in the precipitate was calculated as the difference between the nitrogen in the blank and nitrogen in the supernatant, and was multiplied by 6.25 to give the protein pre-

TABLE I

Agglutination Titer and Specifically Precipitable Protein of Antibody Solutions

Solution	Total protein	Specifically precipitable protein	$\dfrac{\text{Sp. pptble. protein}}{\text{Total protein}}$	Agglutination titer	Ratio $\dfrac{\text{Titer}}{\text{Sp. pptble. protein}}$
	mg. per cc.	*mg. per cc.*	*per cent*		
B II	4.9	2.3	47	1:75 (±)	33
B III	7.1	3.2	45	1:100	31
B IV	4.8	1.9	40	1:60 (±)	32
B V$_A$	14.0	5.5	39	1:200 (±)	36
B VI	32.5			1:400	
B VII	16.5	7.6	46	1:240	32

cipitated. The supernatant was tested for both SSS III and antibody by adding 0.5 cc. 1:20,000 SSS III and 0.5 cc. antibody solution to separate 0.5 cc. samples of the supernatant.

The results are summarized in Table II.

The ratios found in Table II are quite uniform over the fairly wide range of protein concentration from 7.1 to 16.5 mg. per cubic centimeter of antibody solution.* Table III shows that at lower concentrations of protein (and antibody) a given weight of SSS III precipitates somewhat less protein. Very irregular results were obtained with Solution B VI, which was made up only to one-half the original serum volume, and contained 32.5 mg. of protein per cubic centimeter.

* Preliminary data indicate that the effect of variations in pH is small within the range likely to be encountered in the precipitin reaction.

TABLE II

Summary of Analytical Data

Antibody solution	SSS III	Nitrogen			Protein precipitated	Protein Ratio SSS III	Substance in excess
		In blank	In supernatant	In precipitate by difference			
	mg.	mg.	mg.	mg.	mg.		
B III	0	5.79	(5.79)	—	—		
	0.05	5.79	4.79	1.00	6.25	125	Antibody
	0.10	5.79	4.09	1.70	10.63	106	"
	0	5.67	(5.67)	—	—	—	
	0.15	5.67	3.71	1.96	12.24	82	"
	0.20	5.67	3.45	2.22	13.88	69	Both
	0.25	5.67	3.50	2.17	13.56	—	SSS III
	0	5.73	(5.73)	—	—	—	
	0.40	5.73	3.26	2.47	15.44	—	"
	0.60	5.73	3.19	2.54	15.88	—	"
	1.00	5.73	3.19	2.54	15.88	—	"
	2.00	5.73	3.23	2.50	15.63	—	"
	5.00	5.73	3.36	2.37	14.81	—	
	0	5.67	(5.67)	—	—	—	
	5.00	5.67	3.46	2.21	13.81	—	
	20.00	5.67	4.47	1.20	7.50	—	
	50.00	5.67	5.83	No ppt.	—	—	
B V_A	0	11.22	(11.22)	—	—		
	0.05	11.22	10.24	0.98	6.12	122	Antibody
	0.10	11.22	9.39	1.83	11.44	114	"
	0.20	11.22	8.08	3.14	19.63	98	"
	0.25	11.22	7.64	3.58	22.38	90	"
	0.30	11.22	7.14	4.08	25.50	85	Both
	0.50	11.22	6.90	4.32	26.99	54	"
	1.00	11.22	7.05	4.17	26.06	—	SSS III
	2.00	11.22	7.13	4.09	25.56	—	"
	5.00	11.22	7.09	4.13	25.81	—	"
	0.50	11.35	6.97	4.38	27.38	—	
	10.00	11.35	7.89	3.46	21.63	—	
	20.00	11.35	8.40	2.95	18.44	—	
	25.00	11.35	8.90	2.45	15.31	—	
	30.00	11.35	9.28	2.07	12.94	—	
	40.00	11.35	11.15	0.20	1.25	—	
B V_B	0.00	10.21	(10.21)	—	—		
	0.05	10.21	8.77	1.44	9.00	180.0	Antibody
	0.10	10.21	8.09	2.12	13.24	132.4	"
	0.20	10.21	6.96	3.25	20.32	101.6	"
	0.00	10.40	(10.40)	—	—	—	

TABLE II—*Concluded*

Summary of Analytical Data

Antibody solution	SSS III	Nitrogen			Protein precipitated	Ratio $\frac{Protein}{SSS\ III}$	Substance in excess
		In blank	In supernatant	In precipitate by difference			
B V$_B$	*ms.*	*ms.*	*ms.*	*ms.*	*ms.*		
	0.50	10.40	5.72	4.68	29.25	—	SSS III
	10.00	10.40	6.56	3.84	23.95	—	"
	20.00	10.40	7.64	2.76	17.25	—	"
	30.00	10.40	9.00	1.40	8.75	—	"
	40.00	10.40	9.80	0.60	3.75	—	"
	50.00	10.40	10.13	0.27	1.69	—	"
B VII	0.00	13.22	(13.22)	—	—		
	0.05	13.22	12.27	0.95	5.94	119	Antibody
	0.25	13.22	8.58	4.64	29.00	116	"
	0.50	13.22	7.11	6.11	38.19	76	Both
	0.80	13.22	7.11	6.11	38.19	—	SSS III
	1.00	13.22	7.13	6.09	38.06	—	"
B VIII*	0.00	9.40	(9.40)	—	—		
	0.05	9.40	8.39	1.01	6.31	126	Antibody
	0.10	9.40	7.62	1.78	11.13	111	"
	0.15	9.40	6.66	2.74	17.13	114	"
	0.20	9.40	6.24	3.16	19.75	99	Both
	0.25	9.40	6.02	3.38	21.13	85	"

*pH 7.1.

2. *Combination of High* $\frac{Protein}{SSS\ III}$ *Ratio Precipitate with Additional SSS III.*—
5 cc. of antibody Solution B VII, 1 cc. of 1:20,000 SSS III, and 4 cc. of 0.9 per cent saline were mixed and allowed to react as in the preceding experiments (Tube A). On the next day the mixture was centrifuged in the cold, yielding 0.2 cc. of precipitate. Since this contained only about 6 mg. of protein (see Table II) its bulk was composed mainly of entrained supernatant. The supernatant liquid was carefully drained off and 0.2 cc. added to another 15 cc. centrifuge tube (check tube). To each of the tubes 1 cc. of the 1:20,000 SSS III solution was added, the volumes were adjusted to 10 cc. with saline, and the mixtures were shaken mechanically at room temperature for 2 hours and allowed to stand over night in the icebox (Tubes A' and B'). In order to determine whether any effect observed might be due to adsorption rather than to chemical combination, the precipitate from a quantitatively similar Type I pneumococcus antibody-specific substance experiment was treated in the same way with 1 cc. of the Type III SSS solution (Tube C'). The tubes were centrifuged in the cold and 5 cc. from each were mixed

TABLE III
Data on Dilute Antibody Solutions

Antibody solution	SSS	Nitrogen			Protein precipi-tated	Ratio $\frac{Protein}{SSS}$	Substance in excess
		In blank	In super-natant	In pre-cipitate			
	mg.	mg.	mg.	mg.	mg.		
B IV	0.00	3.87	(3.87)	—	—		
	0.05	3.87	3.19	0.68	4.25	85	Antibody
	0.10	3.87	2.78	1.09	6.81	68	"
	0.10	3.87	2.72	1.15	7.19	72	"
	0.15	3.87	2.57	1.30	8.13	54	Both
	0.25	3.87	2.32	1.55	9.69	—	SSS III
	0.50	3.87	2.32	1.55	9.69	—	"
BV$_A$ diluted (2	0.00	3.90	(3.90)	—	—		
cc. made up	0.05	3.90	3.17	0.73	4.56	91	Antibody
to 5 cc.)	0.10	3.90	2.75	1.15	7.19	72	"
	0.15	3.90	2.51	1.39	8.69	58	Both

with 5 cc. of fresh B VII antibody solution (Tubes A″, B″, and C″). Only a slight turbidity developed in the tube containing the supernatant which had been in contact with the high-ratio Type III specific precipitate, indicating that much of the second portion of SSS III added had combined with the precipitate to yield an insoluble product of lower ratio of protein to carbohydrate. Tubes B″ and C″ yielded immediate precipitates. After 2 hours at 37° and letting stand over night in the ice-box the tubes were centrifuged in the cold and nitrogen was determined in the supernatants on 2 cc. aliquots.

Tube	Nitrogen			Protein in precipitate	SSS calculated in precipitate (Protein ÷ 120)*
	In blank	In superna-tant	In precipi-tate		
	mg.	mg.	mg.	mg.	mg.
5 cc. antibody B VII + 5 cc. saline.....	13.79	(13.79)			
(A″) 5 cc. B VII + 5 cc. supernatant from Type III precipitate (Tube A′).	(13.79)	13.58	0.21	1.31	0.011
(B″) 5 cc. B VII + 5 cc. supernatant from check tube (Tube B′).......	(13.79)	13.26	0.53	3.31	0.028
(C″) 5 cc. B VII + 5 cc. supernatant from Type I specific precipitate (Tube C′)......................	(13.79)	13.21	0.58	3.62	0.030

* The rounded-off maximum value in Table II is taken, omitting BV$_B$.

Therefore in the three 10 cc. samples shaken as above with additional SSS III:

	SSS added	SSS recovered above	SSS combined with precipitate
	ms.	*ms.*	*ms.*
(A') Type III precipitate..................	0.05	0.022	0.028
(B') Check tube...........................	0.05	0.056	—
(C') Type I precipitate....................	0.05	0.060	—

It is thus seen that only in the Type III tube did combination occur, and that a high-ratio Type III specific precipitate actually does combine with more SSS III under the conditions of the precipitin test.

DISCUSSION

For purposes of discussion it will be assumed with Felton (*loc. cit.*) that antibody is modified protein, and that, in order to provide a uniform method of measurement, it may be expressed as nitrogen precipitable by specific polysaccharide, multiplied by 6.25. Since only relative values are under consideration, the actual magnitude of the factor used is of little significance so long as it be used throughout. Moreover, Table I shows a correspondence between this measure of antibody content and the agglutination titer, so that its use as a relative measure is independent of the nature of Type III pneumococcus antibodies.

Now it has been shown amply that the reactions of proteins may be explained according to the laws of classical chemistry (7) and it also has been shown that the soluble specific substance of Type III pneumococcus is a salt of a highly ionized poly-aldobionic acid (8). It therefore would appear reasonable to test the experimental data by the law of mass action and thus to determine whether or not the precipitation of the hapten by its homologous antibody shows analogies to the behavior of simpler ionic reactions.

It is evident from Table II that with constant amounts of antibody and increasing amounts of Type III soluble specific substance the ratio of the two components in the specific precipitate changes from approximately 120:1 at the smallest amount of precipitate which can be determined quantitatively with a fair degree of accuracy, to about 60:1 at the point of equilibrium, that is, at the point at which both

antibody and SSS are demonstrable in the supernatant.* It therefore would appear that a small amount of Type III polysaccharide in the presence of much antibody yields a precipitate of the composition 120:1 (in mg.). This is capable of reacting further with increasing amounts of hapten (see Section 2) up to the point at which both components are in equilibrium in solution (*cf.* Table II, last column) when the composition of the precipitate is approximately 60:1. In other words, depending on the relative amounts of the reactants, the specific precipitate is a mixture of varying proportions of two compounds, or a whole series of compounds containing hapten and antibody in varying proportions, whose limits may be expressed as

$$A + S \rightleftarrows AS\ldots(120:1) \ldots\ldots\ldots\ldots\ldots\ldots\ldots(1)$$

$$AS + S \rightleftarrows AS_2\ldots(60:1) \ldots\ldots\ldots\ldots\ldots\ldots\ldots(2)$$

in which S and A are equivalent amounts of Type III specific substance and antibody, respectively, entering into reaction to form the compound of ratio 120:1. A more general expression would be $nA + mS \rightleftarrows A_nS_m$ and $A_nS_m + mS \rightleftarrows A_nS_{2m}$, but is not used to avoid unnecessary complication.

Quantitative support is thus afforded for the contention of Fleischmann and Michaelis (1b) that the specific precipitate may contain the components in multiple proportions. Their objections to Arrhenius' formulation of the precipitin reaction (1a), which was based on the constant composition of the precipitate, are thus fully sustained.

Equation 2 represents an actual equilibrium since a precipitate consisting largely of AS_2 gives up S when shaken with 0.9 per cent saline, the supernatant yielding a fresh precipitate when A is added. That AS can combine with S has been shown in Section 2 of the Experimental Part.

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* It is probable that if the amount of precipitate produced by less than 0.05 mg. of SSS III could have been determined with accuracy ratios of 130:1 and 65:1 or 140:1 and 70:1 would have been found; the more so as in the low-titer or very dilute antibody solutions, in which 0.05 mg. of SSS III is equivalent to a relatively larger amount of antibody, the initial ratios are much lower (*cf.* Table III). However, the present discussion is confined to actual experimental data, and it is hoped that more absolute figures may be supplied at a later date.

The high initial ratios obtained with solution BV_B are inconsistent with the other data and have therefore been disregarded. Had they been included the ratios would have been 130:1 and 65:1 instead of 120:1 and 60:1.

At the point of equilibrium represented by Equation 2 both A and S are present in solution and either may be precipitated by addition of the other. Can this phenomenon, hitherto considered so baffling on account of the known insolubility of the specific precipitate, be quantitatively accounted for according to the law of mass action?

From Equations 1 and 2 may be derived the expression

$$\frac{[A]\,[S]^2}{[AS_2]} = K \dotfill (3)$$

But AS_2 is a sparingly soluble substance and is present in excess at equilibrium, being mainly in the form of a precipitate, hence $[AS_2]$ = a constant and (3) may be written

$$[A]\,[S]^2 = K' \dotfill (4)$$

Moreover, according to (3), addition of either A or S should cause precipitation, and this actually happens, although if appreciable A is added (1) must be taken into account in calculating the composition of the precipitate.

Now although the *molecular* concentrations of A and S are unknown other units may be used provided they are comparable and may ultimately be expressed in terms of molecular concentration. If 1 mg. of antibody protein be called 1 unit of antibody, then the smallest amount of hapten that will combine with it, namely 1/120 mg. may be called 1 unit of specific substance. From Tables II and III it will be seen that, on the average, about 1.5 mg. of A per 10 cc. are present in solution at equilibrium, or 0.15 unit per cubic centimeter. Then the amount of S will be $0.15 \times 2 = 0.3$ units. Substituting in (4), $[0.15] \times [0.3]^2 = K'$ and

$$K' = 0.0135 \dotfill (5)$$

With this value of K′ it should be possible to predict the smallest amount of Type III polysaccharide detectable with an antibody solution or serum of known antibody content. If solution BV$_A$ (Table I) be taken as an average solution, at the 2:3 dilution commonly employed for the precipitin test, the final dilutions would contain 1.1 unit of antibody. Substituting in Equations 4 and 5, $[1.1]\,[S]^2 = 0.0135$, whence $[S] = 0.111$ units, or 0.00089 mg., corresponding to a

dilution slightly greater than 1:1,000,000. This is of the same order of magnitude as the values obtained with whole serum, which are somewhat higher, as would be expected, and range from 1:4,000,000 to 1:8,000,000 (6). It must be remembered, however, that with these proportions of A and S, the composition of the precipitate would be AS, not AS_2. Its solubility, should, however, be of the same order of magnitude. Data on these points will be sought in the near future.

The law of mass action thus supplies an adequate explanation of how appreciable, if small, quantities of Type III soluble specific substance and antibody can exist in solution in the presence of each other, although the solubility of either, especially of the hapten, is greatly diminished when an excess of the other is present.

It can be seen from Table II, however, that maximum precipitation of antibody soon occurs as the concentration of specific substance increases beyond the equilibrium point, after which no further change takes place until at least ten times as much hapten is added as is required to cause complete precipitation (cf. also Sobotka and Friedlander (2c)). The inhibition zone phenomenon* then comes into evidence, but at least a 100-fold excess of hapten over the amount required to reach the equilibrium point is necessary to prevent precipitation completely.†

This solution effect is as specific as the precipitating action of the specific substance, for neither Type I nor Type III specific precipitate will dissolve in a 1 per cent solution (0.9 per cent saline) of Type III or Type I hapten respectively, although either is soluble in 1 per cent homologous hapten solution even after being washed with saline and allowed to stand over night in the ice-box. Moreover, the solution, or inhibition zone, effect is reversible, in that AS_2 separates again when the concentration of soluble specific substance in the solution is reduced by dilution with saline. Therefore it should again be feasible to test the application of the law of mass action to the experimental data obtained in the inhibition zone.

* Owing to the variety of terms in use such as "pre-zone," "pro-zone," or "post-zone" it has been decided to use the term "inhibition zone."

† At certain intermediate concentrations the specific precipitate was insoluble at 0° but redissolved at room temperature or 37° and could be reprecipitated on cooling. This process could be repeated many times. It is hoped to study this effect in greater detail at a later date.

If the precipitate at equilibrium again be considered as AS_2 and no assumption be made as to the composition of the soluble product or products formed, the reaction may be expressed as

$$AS_2 + nS \rightleftarrows AS_p \dots\dots\dots\dots\dots\dots\dots (6)$$

Then

$$\frac{[AS_2] [S]^a}{[AS_p]} = K \dots\dots\dots\dots\dots\dots\dots (7)$$

TABLE IV

Calculation of Approximate Equilibrium Constant for Inhibition Zone

Antibody solution	Maximum protein precipitated AS₂	Protein precipitated in experiment AS₂	Protein dissolved ASₚ	$[AS_p]$ units	Units SSS III added	$[S]$	K' $n = 1$	K' $(\times 10^{-2})$ $n = 2$	K' $(\times 10^{-4})$ $n = 3$
	ms.	ms.	ms.						
B V$_A$	27.4	21.6	5.8	0.58	1200	120	207	248	298
	27.4	18.4	9.0	0.90	2400	240	267	640	1536
	27.4	15.3	12.1	1.21	3000	300	248	744	
	27.4	12.9	14.5	1.45	3600	360	248	894	
	27.4	1.3	26.1	2.61	4800	480	184	883	
B V$_B$	29.3	24.0	5.3	0.53	1200	120	226	272	326
	29.3	17.3	12.0	1.20	2400	240	200	480	1152
	29.3	8.8	20.5	2.05	3600	360	176	632	
	29.3	3.8	25.5	2.55	4800	480	188		
	29.3	1.7	27.6	2.76	6000	600	217		
						Mean: 216			

Since $[AS_2]$ represents the concentration of the difficultly soluble AS_2 in solution, this would be constant at equilibrium provided precipitate were present. Hence, under these conditions

$$\frac{[S]^a}{[AS_p]} = K' \dots\dots\dots\dots\dots\dots\dots (8)$$

The amount of AS_p present can be calculated (as nitrogen $\times 6.25$) by deducting the protein precipitated in the inhibition zone from the maximum precipitable, while free S may, for purposes of calculating n, be taken as the total S present, since the amount in combination is never more than 6 per cent of the total. As in the case of AS_2, 1 mg. of AS_p is considered 1 unit, which cannot introduce a large error, and

1/120 mg. of S = 1 unit, as before.   In Table IV the data obtained in two experiments are calculated in this way and substituted in equation (8), with $n = 1, 2$ and 3.   It will be seen that when $n = 1$ quite constant values of K are obtained.   In Table V is given a more exact calculation, using as [S] in each case the total amount minus the sum of the amount present as AS_2 in the maximum precipitate (Table IV)

TABLE V

Calculation of More Exact Equilibrium Constant for Inhibition Zone. (Alternative for Last Four Columns of Table IV.)   Comparison of Calculated and Found Values of Protein Dissolved

Units SSS III combined			[S]			K'	K' (×10⁻⁴)	K' (×10⁻⁴)	Units protein dissolved in inhibition zone	
$n = 1$	$n = 2$	$n = 3$	$n = 1$	$n = 2$	$n = 3$	$n = 1$	$n = 2$	$n = 3$	Calculated from Equation 8, putting K', 210	Found (Table IV)
55*+6†	67	72	113.9	113.3	112.8	196	221	247	0.54	0.58
64	73	82	233.6	232.7	231.8	260	602	1384	1.11	0.90
67	79	91	293.3	292.1		242	705		1.40	1.21
70	84	99	353.0	351.6		243	853		1.68	1.45
81	107	133	471.9	469.3		181	844		2.25	2.61
59‡+5	70	75	113.6	113.0	112.5	214	241	269	0.54	0.53
71	83	95	232.9	231.7	230.5	194	447	1021	1.11	1.20
80	100	121	352.0	350.0		172	598		1.68	2.05
85	110	136	471.5			185		~	2.25	2.55
87	114	142	591.3			214			2.82	2.76

Mean K' = 210

* Actually maximum units protein × 2 = 54.8.
† Actually 5.8 (Table IV, column 4).
‡ Actually maximum units protein × 2 = 58.6.

plus the additional amount combined in solution.   This is calculated from the dissolved protein (Table IV) putting $n = 1, 2$, and 3, respectively. n still remains equal to 1.   From the last two columns of Table V it is seen that the mean value of K' (Equation 8) so obtained permits, as a check, a fairly close calculation of the protein dissolved in the inhibition zone.

It is therefore suggested that the inhibition zone effect is a chemical equilibrium which may be expressed by simplifying Equation (6) to

$$AS_2 + S \rightleftarrows AS_3 \dots \dots \dots \dots \dots (9)$$

The equilibrium point evidently lies far to the left, since large amounts of S are necessary to cause the formation of appreciable amounts of the compound AS_3.

It therefore appears that the three phases of the precipitin reaction, as exemplified by the soluble specific substance of Type III pneumococcus and its homologous antibody can be quantitatively expressed by the three equilibria:

$$A + S \rightleftarrows \underline{AS} \dots \dots \dots \dots \dots (1)$$

$$\underline{AS} + S \rightleftarrows \underline{AS_2} \dots \dots \dots \dots \dots (2)$$

and

$$\underline{AS_2} + S \rightleftarrows \underline{AS_3} \dots \dots \dots \dots \dots (9)$$

in which the underlined products represent precipitates.

In (1) the reaction tends to proceed strongly to the right, as AS is very difficultly soluble. As the relative concentration of S increases, more and more AS_2 is formed at the expense of the AS, until a new equilibrium is reached. The product AS_2 has an appreciable solubility and dissociation tendency, hence at this point both antibody and specific substance may be detected in solution. Thus, when A is added, there will be a precipitate, since more AS will be formed; when a little S is added, reaction (2) will go further to completion and more AS_2 will be precipitated. When much S is added equilibrium (9) comes into play, and the precipitate redissolves. Moreover, in the three stages of the reaction the proportions of S combined with A vary as 1:2:3.

Thus, in the case of the one specific system under consideration, at any rate, the manifestations of the precipitin reaction may be explained in a very simple manner. Almost any inorganic or organic precipitate, soluble in an excess of the precipitant, might serve as a partial analogy. For example,

$$Ag + CN' \rightleftarrows \underline{AgCN}; \underline{AgCN} + CN' \rightleftarrows Ag(CN)_2'.$$

In this case the first equilibrium would combine (1) and (2) above; solution of the precipitate by excess cyanide would parallel (9), the more so as S is the anion of an acid. Looked at in this light the phenomenon of specific immune precipitation becomes no more—and no less—mysterious than the specificity of silver ion for cyanide ion, or of barium ion for sulphate ion, and must ultimately be traceable to the same underlying causes.*

Whether or not these conceptions are of general application remains to be tested, and work along these lines is under way.

SUMMARY

1. A quantitative study of the reaction between the soluble specific substance of Type III pneumococcus and its homologous antibody has been made.

2. The entire reaction, from excess of antibody, to excess of specific substance with its accompanying inhibition zone effect, may be expressed by three mass-law equations.

3. The significance of these findings is discussed.

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STUDIES IN AGGLUTINATION

IV. The Agglutination Inhibition Zone

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The prozone* or zone of inhibition of flocculation occasionally observed in the higher concentrations of agglutinating sera has been variously explained. This zone is usually seen in old sera although it is sometimes found in fresh sera. When present it may lead to practical difficulties in the serological differentiation of bacteria unless the tests are carried out to the higher dilutions. In our experience the natural zone phenomenon is extremely rare; in fact, in the course of the last few years we have observed almost no agglutinating sera showing this inhibition zone. In an effort to study this phenomenon it has been necessary, therefore, to take advantage of the well known fact that appropriate heating of agglutinating sera produces such a zone of inhibition. The work herewith reported is limited essentially to observations upon artificially produced inhibition zone sera.

Two chief hypotheses have been presented to explain the agglutination prozone.

The older hypothesis suggested by Eisenberg and Volk (1) and supported by Kraus and Joachim (2) accepts Ehrlich's conception of agglutinin as being made up of an antibody-bacteria binding portion (haptophore) and a flocculating portion (zymophore), and assumes that by heating or aging some of the agglutinin is so modified (agglutinoid) that the clumping component is destroyed without, however, affecting the binding portion. As a result agglutinoid may still unite with the bacteria but does not produce flocculation. In order to explain the inhibition effect in high serum concentrations, it is assumed that the agglutinoid in these concentrations has a greater affinity for the bacteria and is, therefore, bound to them to the exclusion of effective agglutinin. To explain the occurrence of flocculation in the higher dilutions it is assumed that the relative proportion of agglutinoid to agglutinin in these concentrations is such that the former is quanti-

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* Owing to the variety of terms used, such as "prezone," "prozone," or "postzone" it has been decided to use the term "inhibition zone."

tatively insufficient to interfere with the effective clumping of the bacteria by the unmodified antibody. The disrepute into which the "haptophore-zymophore" theory of agglutinin structure has fallen has led to the more or less complete discrediting of this explanation.

The second hypothesis is put forward concisely by Zinsser (3) as follows: Agglutinoid zones are analogous to zone phenomena of other antibody reactions, notably the precipitin reaction, and are definitely dependent upon quantitative union between antigen and antibody and have nothing to do with deterioration of antibody by heat or otherwise. In various colloid precipitations in which serum is involved, moderate heating of the serum will strongly reduce its ability to precipitate a suspension. When normal serum is heated it is likely that there is a change in its colloidal state producing a certain amount of colloidal protective property in the serum. In reactions between bacteria and anti-serum it is likely that the antibody carries into union a not inconsiderable amount of active serum constituents. The so-called specific action of agglutinoids is probably due to the fact that the antibody carries into union with the bacteria inactive protein which is colloidally protective by virtue of the heating.

It was with the expectation of producing evidence to substantiate the protective colloid hypothesis that the problem was approached originally in the present investigation. However, as the evidence has accumulated, it has become more and more apparent that the agglutinoid explanation, much modified, more nearly fits the facts, at least as far as the heat induced inhibition zone is concerned.

Methods

Sera Used.—Rabbit and horse agglutinating sera were used. A high titre antityphoid serum (horse) prepared at The Rockefeller Institute in 1917 was obtained from Dr. Chickering. B. melitensis serum was kindly given to us by Dr. Cooper of the New York Board of Health.

Bacterial Strains.—The following organisms were used: B. typhosus, B. melitensis, Type I pneumococcus, and B. dysenteriae, Flexner, Mt. Desert, Shiga, Sonne A and B, and "Y."

Buffers Used.—A glycocoll, sodium phosphate and sodium acetate (G. P. A. mixture) (4) was used in some of the experiments for diluting serum and suspending the bacteria. Most of the experiments were carried out in physiological salt solution.

Washing of Bacteria.—Bacteria were washed at least twice in distilled water in all experiments.

Agglutination Methods.—The macroscopic method was used. Degrees of agglutination are indicated as follows: C., complete, supernatant fluid clear; 3, markedly granular with beginning flocculation; 2, same without flocculation; 1, slightly granular; ±, doubtful agglutination; —, no agglutination.

Potential Measurements.—These were made with the Northrop micro-cataphoresis cell (5).

EXPERIMENTAL RESULTS

Preparation of Inhibition Zone Serum.—When an agglutinating serum is appropriately heated inhibition of flocculation is obtained in the higher concentrations.

A typical heat inhibition zone is shown in Table 1. This is a rabbit antidysenteric serum (Shiga) diluted 1:10 in normal salt and heated for ten minutes at 67°C. In all cases heating of prozone serum was carried out in 1:5–1:10 dilution. Undiluted, or greater concentrations of serum than the above are unsatisfactory for heating as they become turbid and are difficult to work with. It will be observed that no agglutination occurs in the dilutions 1/20 to 1/80 but that it is present above this.

TABLE 1

The Effect upon Agglutination of Heating B. dysenteric (Shiga) Serum at 67°C. for 10 Minutes: a Typical Heat Inhibition Zone Serum

	Agglutination									
	Dilutions of serum									Control
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
Serum unheated (control)...........	C	C	C	C	C	C	C	C	—	—
Serum heated at 67°...............	—	—	—	C	C	C	C	C	—	—

It is important to note that the upper agglutinative titre is practically the same as that of the unheated control serum.

In order to produce this zone phenomenon in different sera, exposure to varying temperatures for the various sera is required.

The minimal point, using short periods of exposure (6–10 minutes), ranged from 62°C., in a Type I pneumococcus serum, to 76°C. for a B. typhoid serum. In some sera we were unable to produce an inhibition zone when using the shorter periods of heating. As the heating point is raised, a level is reached when all agglutination is abolished. This point also is quite variable for the different sera. In our experiments the range was from 64° to 78°C.

The time factor is an important consideration. If sera are heated over prolonged periods at temperatures lower than those noted above, it is possible to produce an inhibition zone.

For example, in the case of a Shiga serum, in which the inhibition zone was obtained by heating for 10 minutes at 66°C., a comparable inhibition zone was produced in 4 hours at 59°C. Table 2 shows the comparison in detail. It will be noted from this table that prolonged heating at this lower temperature (24 hours) has an effect similar to that of the higher temperatures, as noted above, of abolishing all agglutinin. When the time factor is studied for the higher temperatures it is found that this effect is more rapid, being more or less proportional to the temperature. For example, when the serum is heated at 68°C., the inhibition zone begins to appear in 1 minute, is complete in 6 minutes, and practical abolition of all agglutination occurs in 90 minutes.

TABLE 2

The Effect upon Agglutination of Heating Shiga Serum at 59° for 1 to 48 Hours. Shiga Serum Heated at 66° for 10 Minutes Is Shown for Comparison

Time of heating	Agglutination									
	Dilutions of serum									Control
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
Unheated.............	C	C	C	C	C	C	C	C	C−	−
30 minutes.............	C	C	C	C	C	C	C	C	C−	−
1 hour.................	3	C	C	C	C	C	C	C	C−	−
2 hours...............	2	C	C	C	C	C	C	C	C−	−
4 hours................	−	−	3	C	C	C	C	C	2	−
8 hours................	−	−	2	C	C	C	C	C	3	−
24 hours................	−	−	−	−	−	−	±	±	−	−
Heated at 66° 10 minutes...........	−	−	±	C	C	C	C	C	C	−

An interesting demonstration of the effect of varying the heating levels is shown in Table 3.

Separate portions of Shiga serum were heated at temperatures ranging from 55° to 80°C., for 10 minutes. At 63° there is a beginning appearance of the inhibition zone. The zone then widens to reach a peak at 66–69°. Above this the zone narrows to disappear at 72°. Coincident with this narrowing and its loss there is a corresponding drop in the agglutinative titre. At 76° all agglutination disappears. This experiment was done in physiological salt solution; when repeated in a buffer mixture of pH 7.0, similar results were obtained.

A comparable result, though far less marked, was obtained with one other serum. The more usual finding is the appearance of an

inhibition zone immediately succeeded by disappearance of all agglutination as the temperature is further increased.

The findings noted in Table 3 strongly support the hypothesis that the inhibition zone is caused by modification of agglutinin. If one makes the assumption that such heat modified agglutinin (a) still

TABLE 3

Effect of Heating Shiga Serum at Various Temperatures for 10 Minutes

| Temperature of heating | Agglutination | | | | | | | | | Controls |
| | Dilutions of serum | | | | | | | | | |
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
Unheated	C	C	C	C	C	C	C	C—	—	—
55°	C	C	C	C	C	C	C	C—	—	—
56	C	C	C	C	C	C	C	C—	—	—
57	C	C	C	C	C	C	C	C—	—	—
58	C	C	C	C	C	C	C	C—	—	—
59	C	C	C	C	C	C	C	C—	—	—
60	C	C	C	C	C	C	C	C—	—	—
61	C	C	C	C	C	C	C	C—	—	—
62	C	C	C	C	C	C	C	C—	—	—
63	C	C	C	C	C	C	C	C—	—	—
64	±	±	C	C	C	C	C	C—	—	—
65	—	—	C—	C	C	C	C	C—	—	—
66	—	—	—	—	C	C	C	C—	—	—
67	—	—	—	—	C	C	C	C—	—	—
68	—	—	—	—	C	C	C	C—	—	—
69	—	—	—	—	C	C	C	C—	—	—
70	—	—	C—	C	C	C	C—	±	—	—
71	C—	C—	C	C	C	C	±	±	—	—
72	C	C	C	C	C	C—	—	—	—	—
73	C	C	C	C	C	C—	—	—	—	—
74	C	C	C	C	C	C—	—	—	—	—
76	—	—	—	—	—	—	—	—	—	—

retains its binding power although when union has taken place the agglutinin-bacteria complex fails to clump and (b) has a greater affinity for the bacteria than unchanged agglutinin, it follows that the total amount of binding agglutinin in the inhibition zone (*e.g.*, at 66°) will be equal to that of the unheated serum, *i.e.*, the agglutinative titre of the two sera will be the same. This is the fact. Now, when

the heating level is further raised, it will be seen that the inhibition zone is reduced and then disappears.   If we attempt to explain this upon the assumption that this higher heating further modifies the modified agglutinin so that it now loses its binding power, we should expect that the total agglutinative titre would fall off correspondingly. This also is the experimental fact.   Such evidence is indirect rather than direct proof.   It is believed that the experimental findings presented below will give more direct support to the correctness of this view.

Relationship of pH.—Most of the experiments reported were done in physiological salt solution, of pH's varying from 6.0 to 7.0.   That the H ion concentration might be a factor was considered probable. Accordingly, its relationship to the production of the heat inhibition zone was tested at pH's ranging from 5.4 to 7.0.   Table 4 shows the results.   It will be seen that, within the limits of the experiment, a wider prozone is obtained at the higher pH's.   This is essentially the range in which the experiments with salt solution as diluent were carried out.

Specificity of the Inhibition Zone.—In the colloidal protective theory of the inhibition zone it is assumed that heating of the serum changes serum protein so that it has protective properties, and the so-called specific action of agglutinoid is considered to be due to the carrying over of such inactive protein into the union between antibody and organism.   This explanation is usually offered to overcome the stumbling block presented by the older experiments in which it has been shown that absorption of agglutinoid is possible in the inhibition zone.   If colloidally protective inactive protein (*i.e.*, non-agglutinin) is present in an inhibition zone serum it should follow that it might exert an inhibitory effect when added to another, heterologous, serum. Accordingly a suitable inhibition zone serum was obtained by heating an antityphoid serum and this was added, in small to large proportions, to an active, unheated B. melitensis serum.   When the latter was now tested against its homologous organisms, no interference with agglutination in any concentration was noted.   Such findings support the contention that the phenomenon is specific.

In order further to test this hypothesis a similar experiment was performed with very closely related organisms.

TABLE 4

Relationship of pH to Zone of Inhibition in Heat Produced Inhibition Zone Serum. Antityphoid Serum Used

pH	T° of heating	Agglutination											Controls
		Dilutions of the serum											
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	1:20480	
7.0	70	—	—	—	—	—	±	C	C	C	C	C−	—
	71	—	—	—	—	—	±	C	C	C	C	C−	—
	72	—	—	—	—	—	±	C	C	C	C	C−	—
	73	—	—	—	—	—	±	C	C	C	C	C−	—
	74	—	—	—	—	—	±	C	C	C	C	C−	—
	75	—	—	—	—	—	±	C	C	C	C	C−	—
6.6	70	—	—	—	—	3	C−	C	C	C	C	±	—
	71	—	—	—	—	3	C−	C	C	C	C	±	⌐
	72	—	—	—	—	3	C−	C	C	C	C	C−	—
	73	—	—	—	—	3	G−	C	C	C	C	C−	—
	74	—	—	—	—	3	C−	C	C	C	C	C−	—
	75	—	—	—	—	±	3	C	C	C	C	C−	—
6.2	70	—	—	—	—	3	C	C	C	C	C	C−	—
	71	—	—	—	—	3	C	C	C	C	C	C−	—
	72	—	—	—	—	3	C	C	C	C	C	C−	—
	73	—	—	—	—	3	C	C	C	C	C	C−	—
	74	—	—	±	3	C	C	C	C	C	C	±	—
	75	—	—	2	3	C	C	C	C	C	C	C	—
5.8	70	—	—	2	C−	C	C	C	C	C	C−	±	—
	71	—	±	2	C	C	C	C	C	C	C−	±	—
	72	—	2	2	C	C	C	C	C	C	±	±	—
	73	—	2	C	C	C	C	C	C	C	±	±	—
	74	C	C	C	C	C	C	C	C−	±	±	—	—
	75	C	C	C	C	C	C	C	C−	±	—	—	—
5.4	70	—	—	2	C−	C	C	C	C	C	C	±	—
	71	2	C−	C	C	C	C	C	C	C	C−	±	—
	72	2	C−	C	C	C	C	C	C	C	C	±	—
	73	C−	C	C	C	C	C	C	C	C	±	±	—
	74	C	C	C	C	C	C	C	C	±	±	—	—
	75	C	C	C	C	C	C	C	C	±	±	—	—

Shiga serum, diluted 1:9 in normal saline, was heated 10 minutes at 66.5°C. and an inhibition zone obtained. To 0.9 cc. of this Shiga inhibition zone serum, 0.1 cc. each of active (*i.e.*, unheated) Shiga, Flexner, Mt. Desert, Sonne B and "Y"

dysenteric serum was added. This gave proportions of inhibition zone serum to unheated serum of a little more than 1:1. These 1:10 dilutions of the different sera, each with its added component of inhibition zone serum, were now diluted out by halves and homologous organisms were added to each. The results of the experiment are shown in Table 5.

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TABLE 5

Effect of Addition of Shiga Inhibition Zone Serum to Unheated Shiga, Flexner, Mt. Desert, Sonne "B" and "Y" Dysenteric Sera

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The inhibition zone serum was added in an effort to produce a non-specific inhibition zone.

Serum	Organism	Agglutination — Dilutions of the sera										Controls
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
Shiga, unheated	Shiga	C	C	C	C	C	C	C	C	C—	—	—
Shiga, heated	"	—	—	—	C	C	C	C	C	C—	—	—
Shiga, unheated + heated	"	—	—	—	—	C	C	C	C	C	±	—
Shiga, unheated	Flexner	C	C	—	—	—	—	—	—	—	—	—
Flexner, unheated + Shiga, heated	"	C	C	C	C	C	C	C	C	C—	—	—
Shiga, unheated	Mt. Desert	C	C	—	—	—	—	—	—	—	——	—
Mt. Desert + Shiga, heated	"	C	C	C	C	C	C	C	C	C—	—	—
Shiga, unheated	Sonne "B"	±	—	—	—	—	—	—	—	—	—	—
Sonne B, unheated + Shiga, heated	"	C	C	C	C	C	C	C	C	±	—	—
Shiga, unheated	"Y"	±	—	—	—	—	—	—	—	—	—	—
"Y", unheated + Shiga, heated	"	C	C	C	C	C	C	C	C—	—	—	—

It will be seen that in the case of the unheated Shiga serum, the addition of Shiga inhibition zone serum produces a definite zone of inhibition in its higher concentrations; the zone is a little wider here, probably because of the slightly higher proportion of heated to unheated serum as pointed out above. It is worthy of note that the total agglutinative titre is slightly increased, a finding to be expected if the agglutinoid hypothesis is correct. The highly specific nature of the inhibition zone is demonstrated by the failure of the addition

of the Shiga inhibition zone serum to exert any inhibitory effect upon the agglutination of the heterologous organisms by their respective sera.

Absorption Experiments.—The findings of Eisenberg and Volk that absorption of agglutinin (and agglutinoid) occurs in the inhibition zone has been the chief argument in favor of their explanation of the inhibition zone. In order to confirm the work of these investigators the following experiment was carried out.

TABLE 6

Effect of Absorption of Shiga Inhibition Zone Serum with Varying Dosage of Organisms upon Subsequent Agglutination

Dosage of absorbing organisms per cc.	Agglutination after centrifugalization									
	Dilutions of sera									Controls
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
1 billion	—	—	C—	·C	C	C	C	C—	—	—
2 "	—	—	C—	C	C	C	C	C—	—	—
4 "	—	C—	C—	C	C	C	—	—	~	—
8 "	—	C	C	C	C	—	—	—	—	—
*16 "	C	·	·	·	·	·	·	·	·	·
32 "	C	C	C	C	—	—	—	—	—	—
64 "	C	C	C	C	—	—	—	—	—	—
128 "	C	C	C	C	—	—	—	—	—	—
Controls										
Shiga serum, unheated............	C	C	C	C	C	C	C	C	C—	—
Shiga inhibition zone serum (heated)...................	—	—	C—	C	C	C	C	C	C—	—

* Remainder of this experiment lost.

Shiga serum was heated 10 minutes at 67° to produce an inhibition zone. To this serum Shiga organisms were now added in increasing amounts so that the concentrations of the bacteria ranged from 1 billion to 128 billion per cc. The mixtures were then incubated 2 hours in a 37° waterbath and left over night in the icebox. The next day the bacteria were thrown down and the supernatant fluid tested for its agglutinin content. The concentration of the bacteria in this test being 1 billion per cc. The results are shown in Table 6.

It is at once apparent that the findings are in striking agreement with the results reported by Eisenberg and Vook. When the serum

is absorbed with the smaller number of bacteria (1–8 bil./cc.) there is reduction of the inhibition zone and loss of agglutinative titre which is proportional to the concentration of the organisms. Above this, effective agglutinin is absorbed. In other words, it would seem that when the bacteria are relatively fewer in number there is preferential absorption in, or of, the prozone (agglutinoid). This strongly supports the hypothesis that there is present in the inhibition zone serum agglutinin which is so modified that it binds but does not clump bacteria and which has a greater affinity for the organisms than has unmodified agglutinin.

TABLE 7

Effect upon Agglutination, in a Shiga Inhibition Zone Serum, of Varying the Quantity of Bacteria Used for the Test

Number of bacteria per cc.	Agglutination																Controls
	Dilution of the serum																
	1:20	1:40	1:60	1:80	1:100	1:120	1:140	1:160	1:180	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	
16 billion	C	C	C	C	C	C	C	C	C	C	C	C	—	—	—	—	—
8 "	—	C	C	C	C	C	C	C	C	C	C	C	C	—	—	—	—
4 "	—	—	C	C	C	C	C	C	C	C	C	C	C	—	—	—	—
2 "	—	—	—	—	—	—	C	C	C	C	C	C	C	3	—	—	—
1 "	—	—	—	—	—	—	—	—	—	—	C	C	C	C	—	—	—
½ "	—	—	—	—	—	—	—	—	—	—	—	C	C	C	3	—	—
¼ "	—	—	—	—	—	—	—	—	—	—	—	—	C	C	3	—	—
*⅛ "	—	—	—	—	—	—	—	—	—	—	—	—	C	C	3	—	—

* Below this point the suspensions were too light to be read.

In an effort to explain the fact that in an inhibition zone serum, bacteria fail to agglutinate in the low dilutions, but do so in the higher dilutions, the assumption has been made that the relative proportion of modified to unmodified agglutinin is such, in the higher dilutions, that the former is quantitatively insufficient to interfere with effective clumping by the latter. It seemed that this hypothesis should be susceptible of experimental verification. The next experiment (Table 7) seems to supply the necessary proof. If one accepts the assumption that in the lower dilutions of an inhibition zone serum (a) there is present a mixture of agglutinin and agglutinoid (the latter being suffi-

cient in quantity to become an effective factor) and (b) that the agglutinoid has a greater affinity for the bacteria, it follows of necessity, in view of the fact that the agglutinoid must be limited in amount, that when bacteria are added in excess of the number required to unite with all the agglutinoid, unmodified agglutinin will become effective and clumping must result. From Table 7, it will be seen that this is the experimental fact.

When the concentration of bacteria per cc. is 16 billions the condition of excess organisms (over the agglutinoid-binding requirement) is present and agglutination takes place. As the number of organisms drops below this "agglutinoid saturation" point, the inhibition zone appears; and, as the organisms per cc. continue downward their number falls below this saturation level in succeeding dilutions so that with 1/4 billion per cc. in the 1/400 dilution of the serum it falls below such a point and the inhibition zone is present.

These findings have some practical value. Errors in serological diagnostic work due to an inhibition zone may be lessened by having the bacteria in relatively high concentration in setting up agglutination tests. And, conversely, when one is studying the zone phenomenon, the inhibition zone is more readily obtained by keeping the dose of organisms low.

The following experiment was performed in a further effort to test the possible importance, in producing the inhibition zone, of some non-specific, colloidally protective, serum constituent.

Shiga serum was heated and an inhibition zone obtained. The serum was then absorbed by its homologous organisms. This serum now freed of agglutinin and agglutinoid was added to an active unheated serum to see whether there remained in it any non-specific inhibitory factor.

It will be seen from Table 8 that the absorbed serum produces no inhibition zone.

The converse of the experiment above was carried out next, as follows:

Shiga inhibition zone serum was prepared and bacteria in relatively low concentration were added to a 1:20 dilution. They were allowed to incubate for 2 hours in the 56° water bath. The organisms, which had remained unagglutinated (i.e., were stable in suspension), were now centrifuged. They came down in a rather heavy, fluffy mass, more like normally sensitized than unsensitized organisms. The

supernatant fluid was discarded and the bacteria resuspended. Many clumps had appeared following centrifugalization. These were allowed to settle out and the comparatively smooth suspension remaining was tested for agglutinability by addition to active unheated serum. Table 9 shows the result.

TABLE 8

Effect upon Agglutination in a Shiga Unheated Serum of the Addition to This Serum of Absorbed Inhibition Zone Serum

Sera	Agglutination										
	Dilution of the serum										Controls
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	
Unheated serum (control)......	C	C	C	C	C	C	C	C	C	—	—
Heated inhibition zone serum ..	—	—	—	—	~	C	C	C	C	—	—
Absorbed inhibition zone serum.	3	—	—	—	—	—	—	—	—	—	—
Unheated active serum + absorbed inhibition zone serum..	3	C	C	C	C	C	C	C	C	—	—

TABLE 9

Effect upon Agglutination of Bacteria, by Active Unheated Serum, of Previous Sensitization of the Bacteria in an Inhibition Zone Serum of 1:20 Dilution

Sera	Agglutination										
	Dilutions of serum										Controls
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	
Unheated active serum + unsensitized bacteria........	C	C	C	C	C	C	C	C	C	—	—
Heated inhibition zone serum + unsensitized bacteria........	—	—	—	—	C	C	C	C	C	—	—
Active unheated serum + inhibition zone sensitized bacteria	—	—	—	—	—	—	—	—	—	—	—

It will be observed that the organisms sensitized in the inhibition zone are not agglutinated by active serum. In other words, they are saturated with binding, but non-flocculating, modified agglutinin and are, therefore, insusceptible of agglutination by the super-added active serum. Jones (6) working with sera which had been heated to a

higher level than ours, so that practically all agglutinin effect was lost has recently obtained comparable results.

Filtrability Experiments.—In the case of one of our antityphoid heat induced inhibition zone sera (heating was at 74.5° for 12 minutes) it was observed that there was faint opalescence. Attempts were made to centrifuge out the turbidity, without success. Passage through a Berkefeld N filter was then carried out with complete clearing. When this serum was now tested for its agglutinative properties it was found that the inhibition effect had been entirely removed. Table 10 shows the results.

Berkefeld filtration has apparently completely removed the agglutinoid effect. Accompanying this disappearance there is a propor-

TABLE 10

Effect of Berkefeld Filtration of Inhibition Zone Serum upon Subsequent Agglutination

Sera	Agglutination									
	Dilution of the serum									Controls
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
Unheated typhoid serum..........	C	C	C	C	C	C	C	C	C	—
Heated inhibition zone serum.......	—	—	—	—	—	C	C	C	C	—
Filtered inhibition zone serum.......	C	C	C	C	C	C	—	—	—	—

tional drop in the agglutinative titre. From this experiment it is apparent that heat modification of the agglutinin has made it particulate. The fact that upon its removal there is a corresponding drop in agglutinative titre is strong evidence in favor of the fact that this particulate, modified agglutinin still retains its binding power.

This serum was the only one showing perceptible opacity upon heating for the inhibition effect. It was, in fact, the serum which required the highest heating level to produce the zone effect. However, in one of our clear inhibition zone sera, in which filtration showed no effect upon the inhibition or the titre, the addition of kaolin produced identical results as to abolition of the prozone and proportional reduction of titre; kaolin treatment of the unheated serum showed no

effect upon titre. That this is not simple adsorption was demonstrated in an experiment in which treatment of an inhibition zone serum (typhoid) with a heavy dose of heterologous organisms (B. dysenteriae) failed to remove the inhibitory factor.

Cataphoresis Experiments.—It was felt that a study of the charge upon the bacteria when they were sensitized with inhibition zone
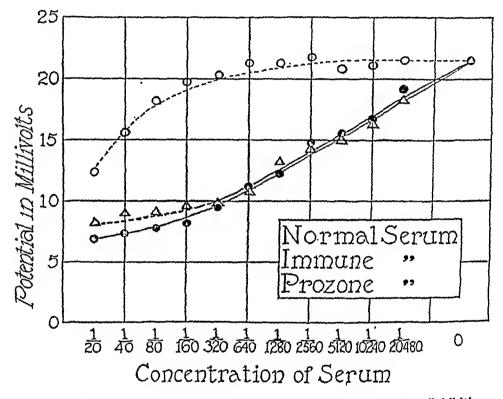
FIG. 1. Effect of unheated agglutinative, and heated agglutinative (inhibition zone) typhoid serum, and of normal serum upon P.D. and agglutination of B. typhosus. Solid line indicates agglutination; broken line, no agglutination. Experiment in G. P. A. buffer mixture, pH 7.0.

serum might be instructive. Accordingly observations were made upon the P.D. of bacteria treated in varying dilutions with normal serum, unheated agglutinating serum, and inhibition zone serum. The results are shown in Fig. 1.

It will be noted from the chart that the charge on the bacteria in the zone of non-flocculation closely approximates that of those which

are sensitized with untreated serum. This is strong evidence that there is union between organism and antibody although there is no clumping. Mudd and his co-workers (7) in their extensive studies of antibody reactions have recently reported similar findings in their observations upon the charge on bacteria in the prozone.

Recently, working with pneumococcus Type III antibody solution, prepared essentially according to the method of Felton,* we have obtained a small though definite inhibition zone after 5 minutes heating at 68°. This antibody solution is almost entirely free of serum protein other than serum globulin, 90 per cent being removed in its preparation (9). This finding is further evidence, although indirect, in favor of the importance, in the production of the inhibition zone, of modified agglutinin (antibody globulin).

DISCUSSION

From the foregoing experiments two points appear clear. (1) Inhibition of flocculation in the inhibition zone is highly specific and this specificity is bound up closely with the presence of agglutinin. (2) The effect is dependent upon modification of the agglutinin, and, agglutinin, so modified, although deficient in flocculating power, has a greater affinity for bacteria than unmodified antibody.

The specificity of the phenomenon is striking. At no time have we been able to demonstrate it to be due to the effect of any inactive (non-antibody) serum constituent which may have become protective as a result of heating. Removal, from an inhibition zone serum, of modified agglutinin (agglutinoid), by specific absorption, Berkefeld filtration, or adsorption with kaolin, has been regularly accompanied by the loss of its inhibitory effect. Hence it has seemed a reasonable conclusion that the inhibitory factor is agglutinoid and not a non-specific, inactive, serum constituent.

It has seemed clear from the experiments reported above that the older observations are correct, namely, that modified agglutinin (agglutinoid) has a greater affinity for bacteria than does unheated antibody. The evidence is striking in the selective absorption experiments above, tables 6–9. To attempt to explain the greater

* The antibody solution was kindly given to us by Dr. Heidelberger and·Dr. Kendall who have been using it in the studies of the precipitin reaction.

affinity of agglutinoid for bacteria is not possible in view of our ignor-
ance of antibody chemical structure. A parallel exists in the greater
avidity of diphtheria toxoid, than toxin, for antitoxin.

That sensitization by unmodified agglutinin and agglutinoid are
very similar is brought out by many of the findings above and espe-
cially by the P.D. determinations. However, although selective
specific sensitization of bacteria by agglutinoid seems to take place,
flocculation does not follow. The use of the "haptophore-zymophore"
hypothesis in explanation of this failure of clumping is not possible as
there is no evidence for such agglutinin structure. However, an
attempt may be made to explain this non-flocculability of the agglu-
tinoid-bacterial complex on the basis of previous studies of the mecha-
nism of bacterial agglutination.

In this work (8) it has been shown that sensitization of bacteria by
agglutinative sera is selective coating by antibody globulin, that by
such film formation at their surface, the bacteria take on the character-
istics of particles of denatured globulin and that subsequent agglu-
tination of the coated bacteria follows the laws governing the floccu-
lation of particles of denatured protein by electrolytes. For effective
selective coating of bacteria by agglutinin it would seem essential
that the antibody be intact. Modification of the agglutinin to
agglutinoid by heating may well alter the agglutinin-globulin complex
sufficiently to interfere with adequate film formation at the bacterial
surface. By virtue of this faulty coating the agglutinoid-sensitized
particles will fail to take on the characteristics of denatured globulin
and flocculation will not occur.

The problem of the rare appearance of an inhibition zone in fresh
sera remains unexplained. It has been shown above that sera are
variably heat sensitive with reference to the production of a prozone.
It is possible that an occasional serum may be heat sensitive at a lower
level than that noted above (59°), that is, within the limits of ordinary
experiments, 56° or less. In support of such a suggested explanation
we have observed one serum (pneumococcus, Type I, horse) in which
an inhibition zone appeared when incubation was at 56° but not at 37°.
It is of interest in this connection that Heidelberger and Kendall (9)
in studying the precipitin reaction have observed flocculation at ice-
box temperatures in mixtures which showed no precipitation at room

temperature. Whenever natural inhibition zone serum is available further investigation will be carried forward.

It is probable that the inhibition zone occasionally observed in old sera is due to some modification of agglutinin comparable to that obtained when serum is heated, *i.e.*, agglutinoid is formed.

CONCLUSIONS

1. The agglutination inhibition zone, artificially produced by heating, has been studied.

2. The phenomenon is specific and is dependent upon the presence in the inhibition zone serum of altered agglutinin (agglutinoid).

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